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RESPIRATION OF SCAB-PRODUCING STRAINS OF ACTINOMYCETES[1]

BY R. J. DOUGLAS AND C. L. SAN CLEMENTE

Abstract

Homogenized suspensions of *Streptomyces scabies* mycelium derived from young cultures were shown to oxidize a number of organic acids. Data indicate that the high autorespiration rate encountered was supported, at least in part, by nitrogenous substances. Manometric data support the concept of tyrosine oxidation through dihydroxyphenylalanine with subsequent melanin formation. The possible role of cell permeability in contributing to the anomalous rate of α-ketoglutarate oxidation is discussed.

Introduction

Potato scab disease has been responsible for an appreciable economic loss to agriculture; control measures in general have been disappointing since the organism appears to be a normal soil inhabitant. A considerable amount of research has been directed toward the scab problem; individual studies have dealt with the disease process as such, possible methods of prevention, and the physiology of the causal organisms. Attempts to control the disease must logically be based on a knowledge of the metabolism of the organisms involved. The present work is concerned with one aspect of this problem and observations are reported on the respiratory activities of suspensions of *Streptomyces scabies* mycelium. Similar investigations of other *Streptomyces* species have recently appeared (2, 5, 8) and the characteristics of actinomycete respiration have been discussed by Schatz *et al.* (11) and by Nickerson and Mohan (9).

Experimental Methods and Materials

The source and identity of the cultures used have been described previously (4). Spores were grown on 6 oz. slants of a glucose–asparagine–salts medium; one slant with confluent growth and heavy spore production yielded about 25 ml. of a dense spore suspension. Spores were inoculated into Difco peptonized

[1]*Manuscript received December 27, 1955.*
Contribution from the Department of Bacteriology, Ontario Agricultural College, Guelph, Ontario, and the Department of Microbiology, Michigan State University, East Lansing, Michigan. Part of a thesis submitted by the senior author to Michigan State University in November, 1955, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[The May number of this Journal (Can. J. Microbiol. 2 : 139-405. 1956) was issued May 3, 1956.]

milk (30 ml./300 ml. flask) and incubated on a reciprocating shaker at 25°–30° C. The mycelium was harvested by centrifugation, and was washed with sodium chloride (0.85%) or potassium chloride (0.2%) solution. Washed mycelium was blended in a glass homogenizer and suspended in *M*/40 phosphate buffer, pH 5.6. The blending procedure reduced the hyphal pellets to a relatively uniform mycelial suspension which will be referred to as a homogenate (see also 8, 11). It should be noted that the extreme cellular disruption characteristic of animal tissue homogenates (10) did not occur. The nitrogen content of homogenates was determined by micro-Kjeldahl analysis.

Conventional manometric techniques (16) were used with a bath temperature of 30° C. and air as the gas phase. Carbon dioxide was absorbed by 0.2 ml. of 20% potassium hydroxide added to the center well. The evolution of carbon dioxide was measured by Warburg's "direct method" with a correction for carbon dioxide retention in the buffer (16).

The amino acids used were L-forms except threonine, valine, leucine, and dihydroxyphenylalanine (dopa) which were racemic mixtures. Amino acids were obtained from the Nutritional Biochemicals Corp., Cleveland, Ohio; non-nitrogenous organic acids were obtained from the British Drug Houses Ltd., Toronto, Ont.

Results

The Effect of Mycelial Age and the Amount of Spore Inoculum on Respiratory Activity

Preliminary experiments indicated that the endogenous respiratory activity of homogenates was very high, and in many cases exogenous oxygen uptake could not be demonstrated. It was found that the ratio of exogenous/endogenous activity was considerably increased when the homogenates were derived from cultures established with massive spore inocula (6.0 ml. of spore suspension/30 ml. medium). The influence of the amount of spore inoculum is shown in Fig. 1. Other experiments indicated that the respiratory activity

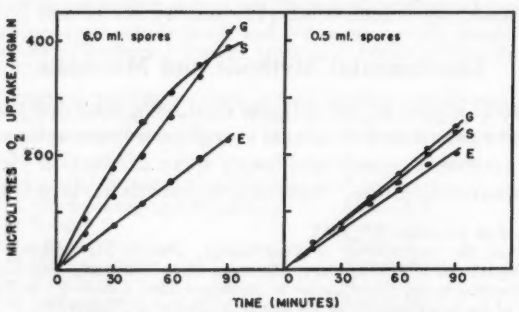

FIG. 1. Respiration of 24-hr. homogenates derived from mycelia established with large and small spore inocula (6.0 and 0.5/flask respectively). E, endogenous, S, succinate, G, glucose. Substrates (5 μM.) added 45 min. before observations begun.

decreased as the mycelium aged. On the basis of these observations young mycelium harvested from cultures established with massive spore inocula was used throughout most of the work.

Endogenous Respiration and the Starvation of Homogenates

During autorespiration of mycelial homogenates the vessel contents were found to become more alkaline and ammonia was produced as determined by direct nesslerization. The R.Q. was found to be about 0.86. Related work showed that extracts of alumina-ground mycelium contained significant quantities of amino acids (3) many of which were rapidly oxidized (*vide infra*). These data suggested that endogenous respiration was supported, at least in part, by nitrogenous substances.

It was consistently observed that autorespiration decreased appreciably after about one hour and then persisted at a fairly uniform rate. A similar observation has been made for *S. nitrificans* grown in a complex medium (11). In the presence of exogenous substrate this rate decrease was not observed (Fig. 2). Homogenates were starved in buffer for one hour on the shaker in most of the work in order to reduce the control (endogenous) activity.

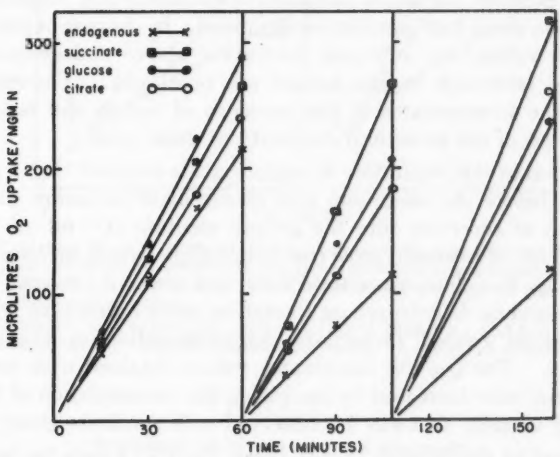

FIG. 2. Nature of the respiration of a 22-hr. mycelial homogenate. Substrates added at 12.5 μM./flask. Manometers opened and reset at 60 and 108 min.

Oxidation of Organic Acids

The oxidation of several important non-nitrogenous organic acids was observed using 17- and 96-hr. homogenates. Substrates were added at a concentration of 12.5 μM./flask. Results are shown in Table I and the oxygen uptake with glucose is included for comparison. All compounds listed were utilized; the rates on succinate and fumarate were of a high order.

TABLE I

RESPIRATION OF YOUNG AND OLD MYCELIAL HOMOGENATES

Substrate*	μl. O_2 uptake† with an homogenate of age: 17 hr.	96 hr.
Endogenous	760	363
Glucose	1256	780
Succinate	1783	704
Fumarate	1574	713
Citrate	1104	386
Acetate	1187	593

**Substrates at 12.5 μM./flask.*
†Values calculated per mgm. N; time period 180 min.

Fig. 3 shows the oxidation of several amino acids by a mycelial homogenate. At the end of the observation period (three hours) the flask contents were treated with trichloroacetic acid (4% final concentration) and, following centrifugation, the supernatants were analyzed for ammonia by direct nesslerization. Data showed that oxygen uptake was considerably in excess of the value expected for simple oxidative deamination and suggested that the residual carbon chain had been further degraded. In the case of phenylalanine, leucine, and valine, no ammonia formation above endogenous could be demonstrated (although oxygen uptake was observed). The relatively low activity of the homogenates in the presence of valine and leucine was of interest in view of the structural similarity of these acids.

Investigation of the respiration of organic acids revealed that a pronounced lag occurred before the maximum rate of succinate oxidation was attained. Incorporation of succinate into the growth medium (1.0 ml. of 5% sodium succinate/30 ml. of medium) gave rise to mycelium which no longer exhibited this delay (Fig. 4). This was not an inductive response, at least in the sense of adaptive succinic dehydrogenase formation, since extracts of non-succinate grown mycelium caused immediate dehydrogenation in the presence of succinate (3). The Q_{O_2} (N) (succinate) values obtained with non-succinate grown material were increased by increasing the concentration of succinate in the Warburg vessels; this was not observed with succinate-grown mycelium.

When tested at the level of 25 μM./flask, Q_{O_2} (N) values for succinate and glutamate were similar (587 and 609 respectively, endogenous not subtracted) and suggested that glutamate was being metabolized through succinate. This suggestion was strengthened by the observation that malonate inhibited the oxidation of both substrates (Table II). Actual oxygen uptake values indicated that the oxidation of succinate was incomplete as was that of glutamate; however, the observed difference [O_2 uptake (glutamate)-O_2 uptake (succinate)] approached the value expected if both compounds were converted to the same end products (observed difference for 5 μM. substrate 120 μl., theoretical 112 μl.).

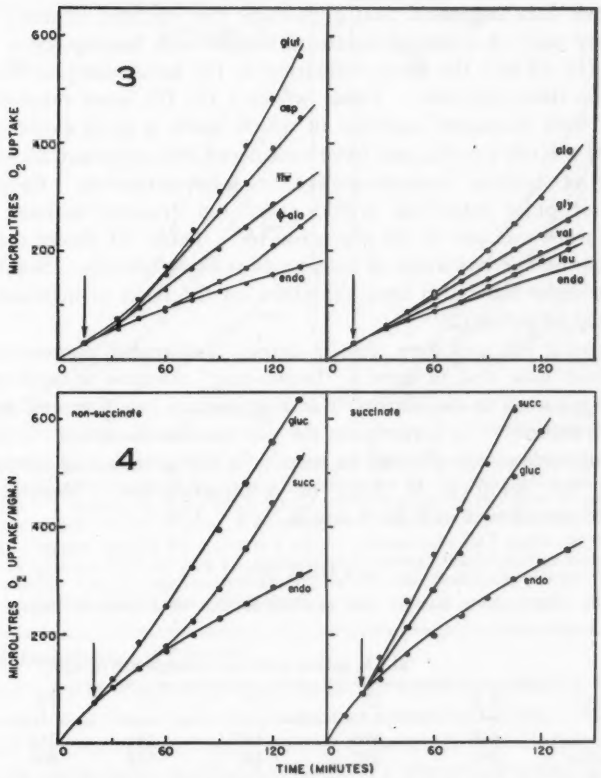

FIG. 3. Oxidation of amino acids by 15.5-hr. homogenate; substrates at 50 μM./flask except phenylalanine (ϕ ala) where 0.5 ml. of a saturated solution was added. Homogenate (0.43 mgm. N/flask) starved one hour in buffer before being added to vessels. Arrows indicate the addition of substrate.

FIG. 4. Oxidation of glucose and succinate (5 μM./flask) by 15.5-hr. homogenates grown in non-succinate and succinate containing media. Addition of substrate indicated by arrows.

TABLE II

INHIBITION OF RESPIRATION BY MALONATE

Substrate*	Q_{O_2} (N)		% inhibition
	No malonate	Malonate†	
Endogenous	100	82	18
Succinate	191	118	38
Glutamate	282	145	49

*Substrates at 5 μM./flask.
†Malonate added at 200 μM./flask.

The above data suggested that glutamate was oxidized through succinate probably by way of α-ketoglutarate although with homogenates of young mycelium (15–18 hr.) the direct oxidation of the latter compound could not generally be demonstrated. Other workers (5, 15) have reported similar difficulties with biological material in which there is good evidence for the existence of a Kreb's cycle, and have considered this apparent anomaly to be the result of cellular impermeability to α-ketoglutarate. Related work showed that older mycelium would carry out transaminations in which α-ketoglutarate was one of the reactants (3). Table III shows the effect of mycelial age on the oxidation of exogenous α-ketoglutarate. Similar results with *S. coelicolor* have also been explained on the basis of increased cellular permeability on aging (2).

Scab-forming cultures were able to cause considerable pigment formation in peptonized milk and to form a "brown-ring" reaction in skim milk (17). This was suggested to be the result of a tyrosinase reaction and subsequent melanin formation. To investigate the mechanism of attack on this amino acid, homogenates were allowed to respire in the presence of tyrosine alone and upon the addition of dopa or α-ketoglutarate. Results of these experiments are shown in Figs. 5 and 6.

TABLE III

INFLUENCE OF HOMOGENATE AGE ON THE DEMONSTRATION OF α-KETOGLUTARATE OXIDATION

	μl. O_2 uptake with an homogenate of age:*					
Substrate	15.5	17	18	21	22	22
Endogenous	449	490	480	384	364	334
α-Ketoglutarate	461	492	486	558	490	485

**Homogenate age in hours. O_2 uptake values are per mgm. N; time period 120 min.*

The induction period prior to the attainment of maximum activity with tyrosine was not reduced by the presence of catalytic quantities of dopa, a condition shown to occur in animal tissue (7). However the data do indicate that the rate of the simultaneous oxidation of tyrosine and dopa is of the order that would be expected if tyrosine were being oxidized through dopa and the concentration of the former were non-limiting. Data in Fig. 6 show that α-ketoglutarate did not stimulate tyrosine oxidation, and chromatographic analyses of the flask contents failed to demonstrate any net glutamate formation. It is probable that for *S. scabies*, unlike some animal tissues (12), transamination is not a requisite step in tyrosine metabolism. Considerable dark pigment was produced in all flasks containing tyrosine and/or dopa.

The above data suggest that the metabolism of tyrosine is predominantly by way of a tyrosinase reaction with the formation of melanin. It must be noted, however, that manometric data constitute a relatively non-sensitive indication of transaminase reactions in the actinomycetes (3).

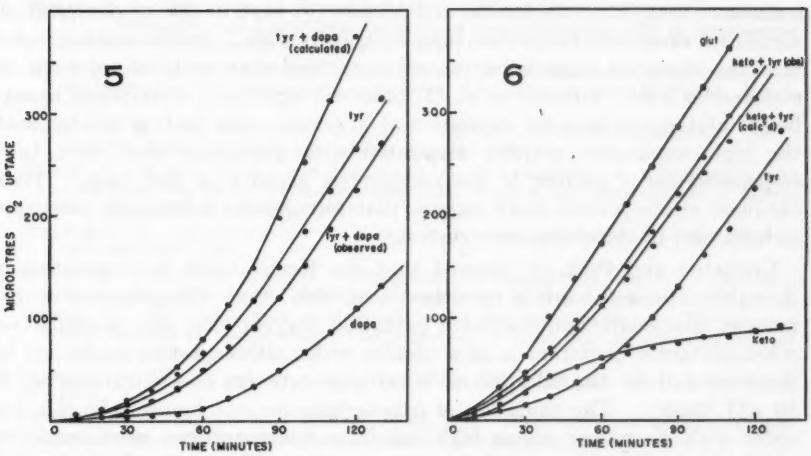

FIG. 5. Oxygen uptake by a starved 22-hr. mycelial homogenate (0.63 mgm. N/flask) in presence of tyrosine (0.25 ml. of a 1% suspension of crystals/flask) and/or dopa (2 μM./flask). Tyrosine + dopa (calculated) curve represents the sum of uptakes when the substrates were added singly. Endogenous uptake (209 μl. at 120 min.) subtracted.

FIG. 6. Oxygen uptake by a starved 22-hr. homogenate (0.7 mgm. N/flask) in the presence of tyrosine (0.25 ml. of a 1% suspension of crystals/flask) and/or α-ketoglutarate (50 μM./flask). Oxygen uptake with glutamate (50 μM./flask) also shown. Endogenous respiration (255 μl. at 120 min.) subtracted.

Action of Dinitrophenol (DNP)

In those cases in which exogenous oxidation terminated during the observation period it was noted that the oxidations were incomplete. Similar results with other organisms have been considered the result of oxidative assimilation (13). Such assimilation is inhibited by DNP; the site of action is believed to be keto acids or their direct precursors (14). When tested at concentrations of 5×10^{-5} and 1×10^{-4} *M*, DNP increased the Q_{O_2} (N) values in the presence of pyruvate and α-ketoglutarate; this increase, however, was not proportionately greater than that which occurred in the endogenous vessels.

The above concentrations of DNP decreased Q_{O_2} (N) values during the oxidation of glucose by unstarved homogenates. This unexpected result was resolved when it was found that increased Q_{O_2} (N) values did occur with starved preparations. In addition, the amount of glucose oxidized was increased to 62% by 5×10^{-5} *M* DNP from a value of 33% in the absence of the compound. It appears that the level of endogenous reserves controls the balance between oxidation and assimilation and the expression of the DNP effect.

Discussion

The high endogenous respiratory activity noted in the present work appears to be characteristic of the actinomycetes (2, 8, 11) and complicates the detection of exogenous repiration. Assimilation rates of a high order may produce

a similar effect (13). Nickerson and Mohan (9) were unable to demonstrate significant exogenous respiration manometrically with *S. fradiae* and suggested that the organism assimilated the substrate and then metabolized from an endogenous pool. Gilmour *et al.* (5) observed significant amounts of loosely bound glutamic acid in the mycelium of *S. griseus*; this finding coupled with the high respiratory activity associated with glutamate may have been responsible for a portion of the endogenous activity of this form. Data obtained in the present work suggest that nitrogenous substances constitute at least part of the endogenous reserves.

Cochrane and Peck (1) showed that the Kreb's cycle was operative in *S. scabies*; present work is consistent with this view. Observations on the rates of glutamate and succinate oxidation suggest that the potential for α-ketoglutarate oxidation is of a similar order although this could not be demonstrated by the addition of α-ketoglutarate (at concentrations up to 50 μM./flask). The oxidation of α-ketoglutarate was found to be of a low order with *S. griseus* unless high substrate concentrations were employed although other evidence pointed to the operation of a tricarboxylic acid cycle (5). Similar inconsistencies with regard to respiratory rates have been reported by Kann and Mills (6) and may be explicable on the basis of membrane permeability.

The high activity in the presence of amino acids is consistent with the recognized capacity of the streptomycetes to form ammonia and produce alkalinity in complex culture media (8). The intense oxidation of a number of the acids (glutamic, aspartic, alanine, threonine, and tyrosine) suggests that these compounds represent a significant potential energy source for the organism.

Acknowledgments

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EXTRACTION, PURIFICATION, AND PROPERTIES OF BACILLUS SOTTO TOXIN[1]

By T. A. Angus[2]

Abstract

A proteinaceous toxin has been extracted from sporulated cultures of *Bacillus sotto* Ishiwata. The toxin is similar in amino acid composition and biological activity to the crystalline inclusions produced by this microorganism and it appears that the toxic protein is associated with the crystals. The toxin is stable, and causes paralysis and death in the larvae of *Bombyx mori* L. (the silkworm) and other lepidopterous insects. The symptoms caused by the toxin are identical with those caused by ingestion of the whole microorganism.

Introduction

The characteristics of *Bacillus sotto* Ishiwata and the localization of a toxic principle inducing paralysis in *Bombyx mori* L. larvae have been described in previous papers (1, 2, 3, 5). The toxic principle appears to be associated with crystalline inclusions produced in sporulating cells of this microorganism (7). In this paper some of the properties of a toxic product derived from sporulated cultures of *B. sotto* will be described. Some of the tests made to determine properties were made also on crystalline inclusions freed of spores (5) and the results of these are discussed.

Toxicity tests were made, as described previously (4), with larvae of *B. mori*, the silkworm.

Experimental

Extraction and Purification of the Toxin

Preliminary Tests to Determine Optimum Conditions

The extraction of the toxic principle of *B. sotto* from sporulated cultures with dilute alkali solutions was described previously (1, 3, 5). Toxic solutions so obtained were straw yellow but could be partially decolorized by prolonged dialysis. By evaporation and freeze-drying of the dialyzed solution a dark brown solid was obtained that was still toxic for silkworm larvae.

A modification of the procedure developed by Hannay and Fitz-James (8) for extracting protein from cultures of *Bacillus thuringiensis* Berliner was used for extracting *B. sotto* toxin. In tests to determine optimum extraction conditions a series of carbonate buffers from pH 9.3 to pH 10.1 were prepared and used as solvents to extract the toxin. It was found that toxic material is released from sporulated cultures of *B. sotto* at about pH 10.0 and above.

[1]*Manuscript received January 20, 1956.*
Contribution No. 269, Forest Biology Division, Science Service, Department of Agriculture, Ottawa, Canada.
[2]*Laboratory of Insect Pathology, Sault Ste. Marie, Ontario. Based, in part, on a thesis submitted to the Faculty of Graduate Studies and Research of McGill University, Montreal, Quebec, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.*

The optimum molarity of solvent for extraction of the toxic principle was determined by suspending equal weights of washed spores and crystals in equal volumes of sodium hydroxide solutions (molarity 0.01 to 0.5 *N*). The mixtures were incubated at 25° C. for three hours with constant shaking, centrifuged at 6000 r.c.f. (7000 r.p.m. Sorvall SS-1A) for 15 min. The supernatant fluids were further clarified by centrifugation, diluted (0–5–25–50–75–100 times), and tested for toxicity. The results (Fig. 1) indicate that the most toxic extract was obtained with 0.02 or 0.05 *N* sodium hydroxide. A possible explanation of the decrease in potency beyond 0.05 *N* is that the toxin is unstable or inactivated in strong alkali.

Chemical analyses showed that desoxyribonucleic acid (DNA) was present in the extracts. Hannay and Fitz-James (8) had included *M* sodium chloride in their procedure in order to remove nucleoprotein and so it appeared that its use should be continued.

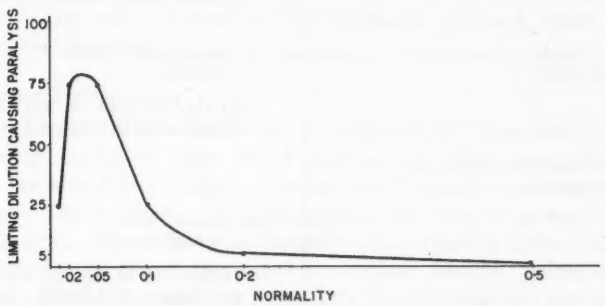

FIG. 1. Extraction of toxic principle of *B. sotto* with sodium hydroxide solution.

Method Adopted for Extracting Toxin

B. sotto grown on nutrient agar at 28° C. for 300 hr. was harvested, washed three times in water at 3° C., once in cold 1 *M* sodium chloride, incubated for one hour at 37° C. in 1 *M* sodium chloride, and then washed three times in cold distilled water. The repeated washings remove the debris of lysed sporangia. The last sediment, consisting principally of spores and crystals, was freeze-dried and weighed. Nine grams of dried spores and crystals were suspended in 1 liter of 0.05 *N* sodium hydroxide solution, stirred for one hour, and centrifuged at 6000 r.c.f. for 15 min. The sediment was re-extracted (three times) in diminishing volumes of alkali (500, 200, 100 ml.) until it was no longer toxic for silkworm larvae. The supernatant fluids were pooled, dialyzed against cold running water for 96 hr., and concentrated to a third of the original volume by evaporation through a dialysis tube.

Acetate buffer, pH 4.1, 1 *M* was added until the pH of the mixture was 4.4 when a dense white precipitate formed. The precipitate was centrifuged and washed three times with distilled water. Toxicity tests indicated that the precipitate was toxic. It still contained nucleoprotein (as DNA); it was therefore resuspended in 1 *M* sodium chloride (1 liter), extracted for 24 hr.

at 3° C., centrifuged, and the insoluble sediment washed three times in double-distilled water. The sediment was redissolved in 0.01 *N* sodium hydroxide solution (200 ml.), shaken for 30 min., and the insoluble material removed by centrifugation. The supernatant fluid was dialyzed against a large volume (six liters) of double-distilled water (at 3° C.), which was renewed every 24 hr. After a week, a flocculent white precipitate began to form. This precipitate was recovered and freeze-dried; the yield was about 180 mgm. A typical extraction is outlined in Fig. 2; at every step of the extraction, toxicity tests were made and the results of these are indicated where appropriate.

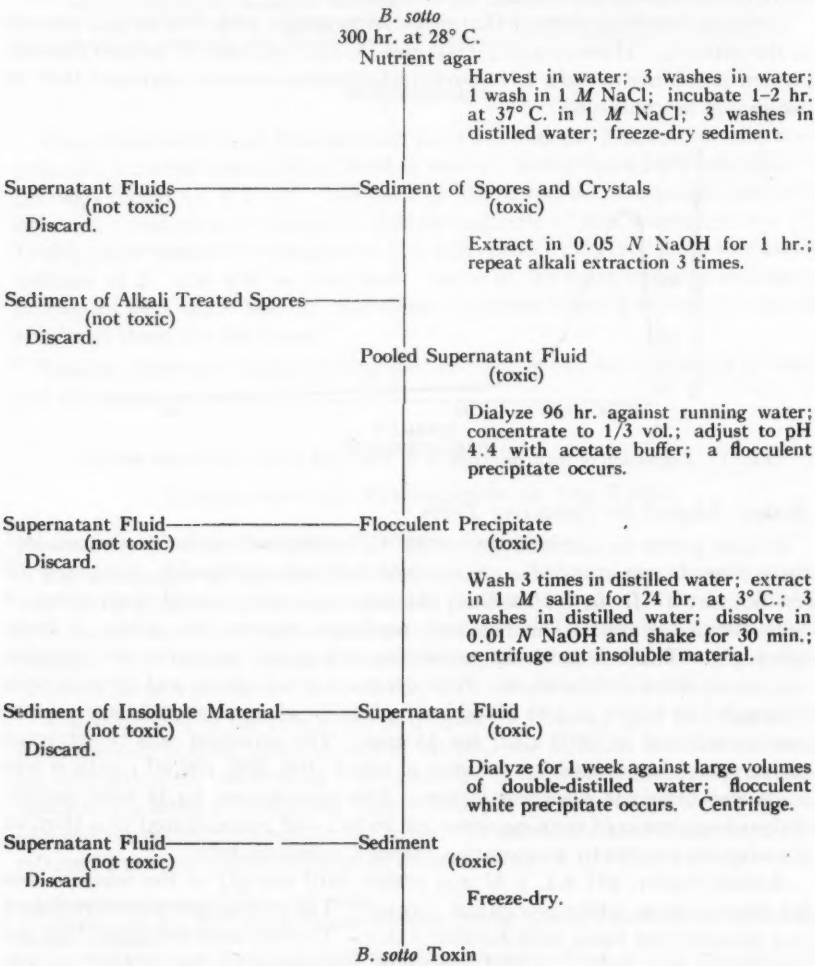
B. sotto
300 hr. at 28° C.
Nutrient agar
Harvest in water; 3 washes in water; 1 wash in 1 M NaCl; incubate 1–2 hr. at 37° C. in 1 M NaCl; 3 washes in distilled water; freeze-dry sediment.
Supernatant Fluids
(not toxic)
Discard.
Sediment of Spores and Crystals
(toxic)
Extract in 0.05 N NaOH for 1 hr.; repeat alkali extraction 3 times.
Sediment of Alkali Treated Spores
(not toxic)
Discard.
Pooled Supernatant Fluid
(toxic)
Dialyze 96 hr. against running water; concentrate to 1/3 vol.; adjust to pH 4.4 with acetate buffer; a flocculent precipitate occurs.
Supernatant Fluid
(not toxic)
Discard.
Flocculent Precipitate
(toxic)
Wash 3 times in distilled water; extract in 1 M saline for 24 hr. at 3° C.; 3 washes in distilled water; dissolve in 0.01 N NaOH and shake for 30 min.; centrifuge out insoluble material.
Sediment of Insoluble Material
(not toxic)
Discard.
Supernatant Fluid
(toxic)
Dialyze for 1 week against large volumes of double-distilled water; flocculent white precipitate occurs. Centrifuge.
Supernatant Fluid
(not toxic)
Discard.
Sediment
(toxic)
Freeze-dry.
B. sotto Toxin

FIG. 2. Extraction of *B. sotto* toxin.

Properties of *B. sotto* Toxin

Toxic material extracted from *B. sotto*, as described above, was subjected to physical, chemical, and biological tests. In the descriptions of the tests and in the various tables presenting the results, the dried material will be referred to as *B. sotto* toxin.

In addition, some of the properties of crystalline inclusions, freed of spores as described previously (5), were determined. The crystals were contaminated with a small number of spores amounting to less than 0.1% of the dry weight of the crystals examined.

Solubility

The crystalline inclusions and the extracted toxin of *B. sotto*, although insoluble in water, are soluble in alkaline solutions (5). Toxin can be precipitated from alkaline solutions by 2% ammonium sulphate without loss of activity. The toxin is insoluble in methanol, ethanol, ether, acetone, chloroform, and benzene.

Electrophoretic Homogeneity

The homogeneity of the toxin was investigated by paper electrophoresis. The following conditions were found satisfactory. Whatman No. 1 filter paper was suspended in an Elphor (Bender and Hobein) apparatus modified to take strips 120 cm. long. A number of strips 2 cm. wide were processed at the same time. The strips were wetted by immersion in buffer and allowed to equilibrate in the closed apparatus for at least 30 min. before the toxin was applied. The buffer used was 0.1 *N* Tris (hydroxymethyl) aminomethane at a pH of 9.0; glycine – sodium hydroxide buffer, 0.1 *N*, pH 9.0, gave equally good results. The toxin (100 μgm. in 0.05 ml. of 0.01 NaOH) was equilibrated for 12 hr. against the buffer and the aliquot applied from a capillary pipette as a band across the strip 8 cm. from the cathode end. A potential difference of 0.3 v. per cm. was maintained resulting in a current of 0.5 ma. At various times (5 to 24 hr.) the strips were removed and dried horizontally in an oven at 105° C. for 15 to 30 min., and fixed and stained for 10 min. in a 0.2% solution of bromocresol green in 95% ethyl alcohol containing 2% glacial acetic acid. The papers were washed in 2% glacial acetic acid (four changes over 20 min.) until the background was free of dye. The papers were dried at room temperature and scanned in a photoelectric apparatus with a slit width of 7 mm. The electropherograms were scanned three times, the readings (as per cent absorption of white light) for each location were averaged, corrected for absorption by the paper as shown by control readings, and plotted.

In all runs, only a single peak was found. A typical run of three strips is shown in Fig. 3. In short runs (5 hr.) a shoulder is apparent but toxicity tests showed that material eluted from the shoulder area was as toxic as that eluted from the peak area. With longer runs (15 and 24 hr.) the shoulder diminished indicating that it was due to tailing. Several electrophoretic

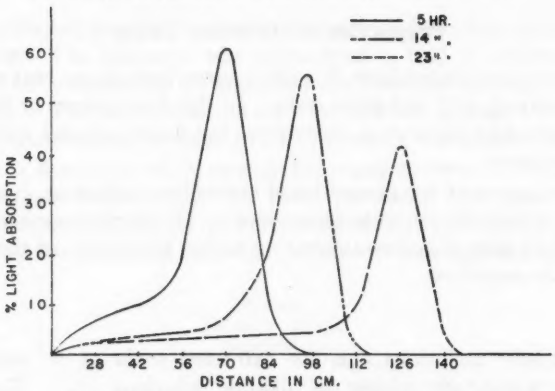

FIG. 3. Electrophoresis of *B. sotto* toxin.

examinations gave similar results and it was concluded that the toxin preparation was electrophoretically homogeneous. Since paper electrophoresis is not a definitive test of purity it would be desirable to have additional tests of homogeneity. Studies of the behavior of the toxin in the ultracentrifuge are under way.

Ultraviolet Absorption Spectrum

The ultraviolet absorption spectrum of the toxin dissolved in 0.01 *N* sodium hydroxide was examined in a Beckman Model DU Spectrophotometer. The observed peak of absorption at 280 mμ (Fig. 4) is typical of proteins. The absence of a peak at 260 mμ indicates that nucleic acid is not an intrinsic component of the toxin. This was also indicated by the fact that impure preparations of toxin containing appreciable amounts of DNA were no more toxic than preparations containing little or no DNA.

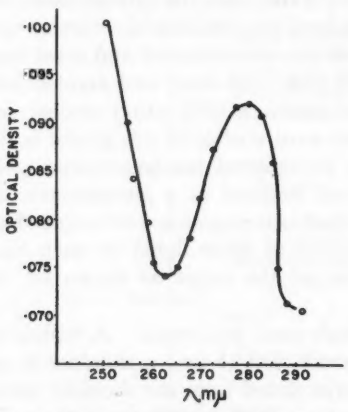

FIG. 4. Ultraviolet absorption spectrum of *B. sotto* toxin dissolved in 0.01 *N* sodium hydroxide.

Nitrogen and Phosphorus Content

A nitrogen estimation made by a modified Nessler's method showed that the toxin contained 17.8% nitrogen; the crystals contained 18.0% nitrogen.

Phosphorus was determined by the method of Bergold and Pister (6). The toxin contained no measurable phosphorus; the crystals contained 0.05% phosphorus. The values obtained for *B. sotto* crystals are thus very similar to those obtained by Hannay and Fitz-James (8) for the crystals of *B. thuringiensis* (17.5% N and 0.000% P).

Amino Acid Composition

A quantitative chromatographic analysis of an acid hydrolyzate of *B. sotto* toxin was made. The method used was based on that developed by Wellington (10) and modified by Wyatt *et al.* (11). The results are given in Table I, which also includes the results of a similar analysis of the amino acid composition of crystals. The toxin and the crystals have very similar amino acid compositions. The values for the toxin are based on the results of three determinations. Only small amounts of crystals were available for study, so only one determination was made. The values for the crystals are subject to the error introduced by the presence of a small number of spores in the crystal suspension.

TABLE I

AMINO ACID COMPOSITION OF *B. sotto* TOXIN AND CRYSTALLINE INCLUSIONS*

Amino acid	Crystalline inclusions	Toxin	
		Average	Range
Arginine	9.4	9.6	9.5– 9.7
Lysine	4.2	3.6	3.5– 3.9
Cysteine and/or cystine	1.1	1.2	1.2– 1.3
Histidine	1.7	2.7	2.7– 2.8
Aspartic acid	9.5	9.6	9.3–10.2
Glutamic acid	12.9	11.8	11.6–12.0
Glycine	2.7	3.2	3.1– 3.3
Serine	5.6	4.8	4.7– 4.9
Alanine	3.2	2.8	2.7– 2.9
Proline	6.7	7.5	7.4– 7.6
Tyrosine	3.9	6.8	6.6– 7.0
Threonine	5.2	4.5	4.3– 4.7
Methionine	0.6	1.3	1.3– 1.4
Phenylalanine	7.4	8.6	8.5– 8.7
Valine	5.0	5.3	5.2– 5.4
Leucine and/or isoleucine	10.4	11.2	11.2–11.3
Tryptophan†	2.1	2.6	2.5– 2.7
Total	91.6	97.1	

**Estimated by paper chromatography of acid hydrolyzates and expressed as grams of amino acid residues per 100 gm. of protein analyzed.*

†Tryptophan was determined separately.

Effect of Temperature and Storage

A test was made of the effect of heating a suspension of *B. sotto* toxin in water, a solution of the toxin in 0.01 *N* sodium hydroxide, and a suspension of crystalline inclusions in water. Two milliliter aliquots of each were heated in a water bath at various temperatures for 10, 20, 30, and 60 min. The aliquots were cooled and tested for toxicity. An unheated suspension was used as a control. The results (Table II) indicate that the toxin is inactivated on exposure to heat whereas crystals suspended in water are much more resistant.

Crystals from *B. sotto* cultures were stored in various ways, and at regular intervals examined in nigrosin films and by dark-field microscopy. The treatments included storage as a suspension in water or saline at 3° C., or at 21° C., up to 12 months; storage as a freeze-dried powder for six months at 3° C.; and as an air-dried spore powder preparation (9) in an evacuated desiccator containing silica gel for 18 months at 3° C. None of these treatments had any apparent effect on the form of the crystals or on their toxicity.

Solutions of *B. sotto* toxin in dilute alkali (0.01 *N*) or suspensions of the toxin in water were still toxic after six months' storage.

Effect of pH

The isolation and purification of *B. sotto* toxin by extraction at pH 11.0 to 12.0 with sodium hydroxide and precipitation at pH 4.0 to 4.5 with acetate buffer indicated that the toxin was stable over a wide pH range. Tests with citrate buffer (0.1 *M*) at lower pH values showed that a toxic flocculent precipitate occurred at values down to pH 3.3. Below this value precipitation no longer occurred and the preparation was not toxic. These results indicate that the toxin is stable from about pH 3.3 to pH 12.0.

TABLE II

EFFECT OF HEAT ON THE TOXICITY OF *B. sotto* TOXIN AND CRYSTALLINE INCLUSIONS

		Effect on toxicity of		
Temperature, ° C.	Time in min.	Toxin dissolved in alkali	Toxin suspended in water	Crystals suspended in water
50	10	Toxic	Toxic	Toxic
	20	Toxic	Toxic	Toxic
	30	Inactivated	Toxic	Toxic
	60	Inactivated	Toxic	Toxic
60	10	Inactivated	Toxic	Toxic
	20	Inactivated	Toxic	Toxic
	30	Inactivated	Inactivated	Toxic
	60	Inactivated	Inactivated	Toxic
70–90	10–60	Inactivated	Inactivated	Toxic
100	10	Inactivated	Inactivated	Toxic
	20	Inactivated	Inactivated	Toxic
	30	Inactivated	Inactivated	Inactivated
	60	Inactivated	Inactivated	Inactivated

Effect of Organic Solvents

The study of the solubility of *B. sotto* toxin in various organic solvents (see above) also provided information on the stability of the toxin in these compounds. It was found that the dry toxin is stable in the presence of methanol, ethanol, ether, acetone, chloroform, or benzene.

In another experiment, six different organic solvents (5 ml.) were mixed with 1 ml. of a toxin solution (0.1% in 0.01 *N* NaOH), shaken thoroughly, incubated overnight at 22° C., shaken thoroughly for five minutes, the solvent evaporated under reduced pressure, and any residue tested for toxicity. It was found that following this treatment *B. sotto* toxin was inactivated by ethanol, methanol, acetone, and chloroform but not by benzene or ether.

Other Tests of Stability

If the dried toxin is dissolved in dilute alkali (0.01 *N* NaOH), the resulting solution can be diluted with water without precipitation of the toxin. The toxin is stable in dilute solutions; a series of dilutions of a 1% alkaline solution of the toxin to as low as 0.01% retained their activity after three months at 3° C. The toxin is inactivated by mercuric chloride, trichloracetic acid, formaldehyde, and iodine.

Toxicity for Insects

Tests with B. mori (the Silkworm)

The potency of the toxin was tested, using the methods described previously (4). From tests of four different lots of toxin an average LD_{50} of 0.5 μgm. of toxin per gram larva was obtained. The results of a typical test are presented in Table III.

In Table IV are shown the results of a test of toxicity by injection. The toxin, dissolved in 0.01 *N* NaOH, was injected with a microsyringe into the body cavity through an abdominal proleg. Although the control insects that ingested an equal amount of the same preparation became paralyzed, those receiving injections of the toxin were not affected. This is unusual since most toxins are active when injected into a suitable host.

TABLE III

TOXICITY OF *B. sotto* TOXIN FOR SILKWORM LARVAE

Toxin in μgm. per gram larva	No. of larvae	Paralysis (P) or death (D) in:			% mortality
		4 hr.	18 hr.	24 hr.	
5	10	10 P		10 D	100
2.5	10	10 P		10 D	100
1.5	10	10 P		10 D	100
1.0	20	10 P		10 D	100
0.5	20	4 P	5 P	7 D	35
0.25	20	4 P	4 P	4 D	20

TABLE IV

TOXICITY OF *B. sotto* TOXIN FOR SILKWORM LARVAE

Dose per larva	Method of dosing	No. of larvae	Paralysis (P) or death (D) in: 4 hr.	20 hr.	120 hr.
1 μgm.*	Ingestion	10	10 P	10 D	10 D
Solvent control	Ingestion	10	0 P	0 P	0 D
1 μgm.*	Injection	10	0 P	0 P	0 D
Solvent control	Injection	10	0 P	0 P	0 D

**1 μgm. of toxin in 0.005 ml. of 0.01 N NaOH.*

Tests with Other Insect Species

The pathogenicity of *B. sotto* for the larvae of several native Canadian insect species has been reported previously (2). The toxicity of *B. sotto* toxin for the larvae of these species was also studied and the results will be presented in a subsequent paper. Briefly, all of the lepidopterous species susceptible to infection by the whole microorganism were also affected by the toxin.

Discussion

When the results of the various analyses are considered there is no doubt that the toxin and the crystalline inclusions of *B. sotto* are proteinaceous. This agrees with the findings of Hannay and Fitz-James (8) for the crystalline inclusions of *B. thuringiensis*. The amino acid analyses of *B. sotto* toxin and crystals are quite similar. It was earlier shown (5) that *B. sotto* crystalline inclusions cause paralysis and death in silkworm larvae; the toxin provokes identical symptoms and it too is lethal for the silkworm.

The association of the toxic principle of *B. sotto* with the crystals is supported by the following evidence:

1. Only sporulating cultures contain crystalline inclusions and only sporulating cultures cause paralysis in larvae (5).
2. Crystals alone fed to larvae result in paralysis (5).
3. If in a mixture of spores and crystals the latter are removed by treatment with alkali, the spores alone are harmless when ingested but the alkaline solution of crystals causes paralysis of larvae (5).
4. The amino acid composition of the toxin and crystals is very similar.
5. The symptoms in susceptible larvae treated with toxin or crystals are identical.
6. Only those strains of insect pathogens which produce crystalline inclusions cause paralysis in silkworm larvae; these include *B. sotto*, *B. thuringiensis*, and *B. cereus* var. *alesti* Toumanoff and Vago.

It seems therefore reasonable to conclude that the paralysis-inducing principle is associated with or derived from the crystals. It is not certain that the crystals are composed entirely of toxic protein. Adequate proof of

this requires a refinement of the method of testing toxicity; such studies are in progress.

There is some evidence that the toxin is partially inactivated by strong alkali. The results given in Fig. 1 show that more potent extracts are obtained with mild than with strong alkali solution; those in Table II that the toxin is more easily inactivated when in solution than it is in the crystal. The assumption that the toxin is partially denatured or inactivated during extraction is supported by the low yield. The LD_{50} of whole *B. sotto* (spores, crystals, and debris) is about 1.5 μgm./gm. (4) and that of sotto toxin is 0.5 μgm./gm. In the extraction described above, from a potential of 6,000,000 LD_{50} (9 gm.) only 400,000 LD_{50} (180 mgm.) were obtained, a recovery of about 7%. Although there is some mechanical loss in washing, resuspending and so on, it appears that inactivation is responsible for the major loss of activity.

The ineffectiveness of the toxin when injected into the body cavity of silkworm was unexpected and in this respect *sotto* toxin is unlike the classic bacterial toxins. Repeated attempts have been made to induce paralysis by injection in various forms but without success; all the preparations were toxic orally. There are a number of possible explanations; the toxin is a protoxin which must be either activated or converted in the gut to an active compound; the sensitive site or receptor site is exposed only in the gut; the toxin is inactivated or inhibited by some component of the blood. The idea that the sensitive site may not be present or at least exposed in the body cavity is supported by the observation that blood from paralyzed larvae when injected into the body cavity has no effect.

A number of problems concerning *B. sotto* remain to be solved. From the point of view of insect pathology, the most important is the mode of action of the toxin. It is hoped that since a uniform toxin preparation is now available for use such studies will be simplified. Some preliminary experiments have been made and the work is continuing.

Acknowledgments

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A COMPARISON OF THE OXIDATIVE METABOLISM OF LIGHT AND DARK GROWN RHODOSPIRILLUM RUBRUM[1]

By P. G. Crook[2] and E. S. Lindstrom

Abstract

Using a manometric or a dehydrogenase assay, activity for most of the intermediates of the tricarboxylic acid cycle was demonstrated in extracts from *Rhodospirillum rubrum* grown photosynthetically (anaerobic light), heterotrophically (aerobic dark), or a combination of both (aerobic light). Dehydrogenases for succinate and α-ketoglutarate were more active in extracts from cells grown photosynthetically. Extracts from cells grown heterotrophically had the greatest oxidative activity. Succinic dehydrogenase was associated with larger macromolecular units in all extracts while the other dehydrogenases behaved as soluble enzymes. Conditions of cultivation affected the rate at which keto acids were formed from citrate. *Rhodopseudomonas palustris* also possessed tricarboxylic acid dehydrogenases whether grown photosynthetically or heterotrophically.

Introduction

Rhodospirillum rubrum is one of the several members of the *Athiorhodaceae* that possesses a photosynthetic metabolism as well as a dark aerobic heterotrophic metabolism (12). Conditions of cultivation (light vs. dark growth) have been shown to influence the macromolecular organization in *R. rubrum* (9). Several workers have compared the fermentative or synthetic metabolism of photosynthetically and heterotrophically grown *Athiorhodaceae* (4, 7, 10). The enzymes of the tricarboxylic acid cycle have been demonstrated in light grown *R. rubrum* (2, 13). To determine the influence of the growth environment on the metabolic organization of the cell, *R. rubrum* grown photosynthetically or heterotrophically was compared for differences in variety, activity, and distribution of the tricarboxylic acid cycle dehydrogenases.

Experimental Methods

For growth in the light under anaerobic conditions, *R. rubrum* S-1 was handled essentially as outlined previously (14). For growth under aerobic conditions, cultures were grown in cotton-stoppered Erlenmeyer flasks (100 ml. medium in 500 ml. flask) on a reciprocating shaker either under a bank of incandescent lamps (aerobic light) or in a light tight box (aerobic dark). Cultures were incubated five days at 25° C. in the light and seven days in the dark. Cell paste was suspended in 0.05 *M* phosphate buffer pH 7.5 (20% wet weight of cell paste/vol. of buffer) and treated for three minutes in a 10 kc. Raytheon magnetostriction oscillator at 3°–5° C. This brei was centrifuged at 15,000 × *g* for 15 min. at 3° C. and the supernatant used immediately or stored at −20° C. Organic acids were qualitatively detected

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Contribution from the Department of Bacteriology, the Pennsylvania State University, University Park, Pennsylvania, U.S.A.
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using one dimension paper chromatography (1). Details of other assay procedures are given with the respective tables. Substrates were obtained from the usual commercial sources and were dissolved in water double-distilled from glass.

Results and Discussion

Data in Table I indicate that extracts obtained from cells under all three conditions of growth possessed the dehydrogenases for the tricarboxylic acid cycle. Extracts from cells grown anaerobically in the light (photosynthetically) had about twice the activity on succinate and α-ketoglutarate of those grown heterotrophically. All these measurements were made in subdued light to preclude photooxidation. The reactions from succinate to malate were also demonstrated using paper chromatography while α-ketoglutarate appearance from citrate and disappearance to succinate were demonstrated by colorimetric measurements of the phenylhydrazones. The expected malonate inhibition was observed with the succinic dehydrogenase derived from cells grown under the three different conditions.

Data in Table II show that extracts from cells grown under all three conditions were able to oxidize intermediates of the tricarboxylic acid cycle. The extracts from cells grown aerobically in the dark possessed greatest

TABLE I

DEHYDROGENASE ACTIVITY OF EXTRACTS OF *R. rubrum*

Conditions of cultivation	Specific activity* with indicated substrate				
	Citrate	Ketoglutarate	Succinate	Fumarate	Malate
Aerobic dark	0.05	0.08	0.1	0.01	0.01
Aerobic light	0.05	0.05	0.1	0.01	0.03
Anaerobic light	0.04	0.2	0.2	0.005	0.01

** Per cuvette: 10 μM. substrate; 20 μM. phosphate buffer pH 7; 0.15 μM. 2,6-dichlorophenol indophenol; 20 μM. KCN pH 7; extract; water to 3.0 ml., 25°C. Change in optical density at 600 mμ measured in Beckman spectrophotometer. Blank values subtracted. Specific activity equals change in O.D. per 10 min. per 0.05 ml. extract.*

TABLE II

OXIDATIVE ACTIVITY OF EXTRACTS OF *R. rubrum*

Conditions of cultivation	μl. O_2 uptake per 60 min. with indicated substrate				
	Citrate	Ketoglutarate	Succinate	Fumarate	Malate
Aerobic dark	200 –	250	220	48	100
Aerobic light	60	50	170	35	38
Anaerobic light	150	170	120	0	0

Per Warburg cup: 10 μM. substrate; 20 μM. phosphate buffer pH 7; 700 μM. KOH (center well); 2.0 ml. extract; total volume 3.0 ml. at 30°C. Blank values subtracted.

oxidative activity. In order to demonstrate oxidative activity from extracts of cells grown anaerobically in the light, young cultures (three to four days' incubation) had to be used, and a blank oxygen uptake was observed here (50 μl./hr.) that was not observed with the other extracts. The aerobically grown cells all possessed a cyanide sensitive oxidase while less stimulation was observed when cyanide was added to the dehydrogenase assay using extracts from anaerobically grown cells.

Previous work has shown that the reducing power generated by light may or may not affect a specific metabolic area of *R. rubrum*. Photosynthetically grown *R. rubrum* assimilated acetate or carbon dioxide by essentially similar reactions in the light or dark (4). Acetone was metabolized similarly in the light or dark by heterotrophically grown *Rhodopseudomonas gelatinosa* (10). Heterotrophically grown *R. rubrum* anaerobically decomposed pyruvic acid in the dark yielding the products of a typical propionic fermentation, but in the light H_2 and CO_2 were the products (7). Similar results were obtained with photosynthetically grown *R. rubrum*. Light markedly stimulated N_2 fixation by photosynthetically or heterotrophically grown *R. rubrum* (8). The tricarboxylic acid cycle dehydrogenases were demonstrated with dried cell preparations of *R. rubrum*, but these cells were grown semianaerobically in the light and then shaken overnight in buffer to deplete the endogenous substrates (2), so the cells were subjected to a changing environment. Similar results have been obtained with sonic extracts from *R. rubrum* grown anaerobically in the light (13). A light induced phosphorylation was demonstrated using a particulate fraction from light grown *R. rubrum* (3). Our data indicated that growth in the presence or absence of light did not affect the qualitative presence of tricarboxylic acid enzymes, but conditions of cultivation affected the apparent level of activity on α-ketoglutarate and succinate.

In Table III data are presented showing the distribution of the dehydrogenases in extracts of cells grown anaerobically in the light or aerobically in the dark. The dehydrogenases for citrate, α-ketoglutarate, fumarate, and malate were apparently soluble enzymes, and conditions of growth had no influence on their distribution. With succinic dehydrogenase the activity was apparently associated with macromolecular units (chromatophores) of relatively high molecular weight (14). Conditions of cultivation had an apparently quantitative effect on the distribution of succinic dehydrogenase within the cell, more activity being sedimented by both centrifugal fields with extracts from cells grown photosynthetically. The pigment bearing chromatophores have been reported absent in *R. rubrum* grown aerobically in the dark though the dark grown cells did have a considerable component with a sedimentation constant of 55 Svedbergs in contrast to the chromatophores in light grown cells with a sedimentation constant of 153 S (9). Our data indicated that the succinic dehydrogenase of light grown cells was borne on larger more easily sedimentable particles than the succinic dehydrogenase of dark grown cells.

TABLE III

DISTRIBUTION OF DEHYDROGENASE ACTIVITY

Conditions of cultivation	Treatment*	Fraction tested	Per cent of initial activity†		
			Citrate or ketoglutarate	Succinate	Fumarate or malate
Aerobic dark	25,000 × *g*	Supernatant	100	95	100
		Sediment	0	1	0
	144,000 × *g*	Supernatant	100	59	100
		Sediment	0	72	0
Anaerobic light	25,000 × *g*	Supernatant	100	80	100
		Sediment	0	40	0
	144,000 × *g*	Supernatant	100	36	100
		Sediment	0	121	0

* *Centrifugal field for 60 min. at maximum radius (8.1 cm.) in Spinco Model L Centrifuge.*
† *Based on activity of complete extract using indophenol assay.*

As photosynthetic growth apparently increased the rate at which α-ketoglutarate and succinate were dehydrogenated, additional information was accumulated to determine the mechanism causing the differences. Data in Table IV indicate that conditions of cultivation influenced the keto acid metabolism when changes in keto acid concentration were measured, rather than the rate of reduction of indophenol. At the end of the assay period with citrate as the substrate, more α-ketoglutarate was present using extracts from anaerobic light cells than from aerobic dark cells. Cultural history of the extracts had greater influence on keto acid metabolism than did the presence of light or air during the assay period. The other differences observed in the rate of keto acid metabolism were consistent though of doubtful significance.

A comparison of the data in Tables I, II, and IV revéals consistencies as well as certain inconsistencies. Extracts of cells grown under all three conditions were able to metabolize intermediates of the tricarboxylic acid cycle. Anaerobic light grown cells have been shown to oxidize fumarate and malate if the pH is shifted to 9 (2). A comparison of the activities on the various substrates between Tables I and II is of questionable significance as different reactions were being measured. The anaerobic light extracts possessed greatest dehydrogenase activity, but their oxidative activity was apparently limited by the lack of terminal oxidases as a result of their anaerobic cultivation. A comparison between Tables I and IV is difficult as in Table I a rate reaction was being measured while in Table IV an equilibrium value was being measured. The data can be compared using the same assay or the same method of cultivation, and the results of such comparisons show that the methods of cultivation affected the levels of activity but not the occurrence of the various enzymes of the tricarboxylic acid cycle.

No coenzymes were added to the reaction mixtures since the purpose was to compare the actual, not the potential activity of the various dehydrogenases

TABLE IV

KETO ACID METABOLISM BY *R. rubrum*

Conditions of cultivation	Conditions of assay	μM. Keto acid*	
		Appearing†	Remaining‡
Aerobic dark	Aerobic light	0.2	0.09
	Aerobic dark	0.2	0.09
	Anaerobic light	0.3	0.07
	Anaerobic dark	0.3	0.07
Anaerobic light	Aerobic light	1.1	0.07
	Aerobic dark	0.9	0.08
	Anaerobic light	1.0	0.07
	Anaerobic dark	0.8	0.07

* *Determined by method of Koepsell and Sharpe* (6).
† *10 μM. citrate; 25 μM. phosphate buffer pH 7; 0.3 μM. 2,6-dichlorophenol indophenol; 0.4 ml. extract; water to 3.0 ml., 25°C. for 20 min.*
‡ *Same as above except 0.2 μM. α-ketoglutarate instead of 10 μM. citrate.*

in the several types of extracts used. The coenzyme level has been shown to be an important intracellular control mechanism (11), and the addition of external amounts could mask a difference in the actual activity among the extracts studied.

When the spectra of extracts of cells grown under various growth conditions were compared, it was noted that the extracts of aerobically grown cells possessed less chlorophyll and carotenoid pigments than did extracts from anaerobically grown cells. Our data indicated that oxygen tension apparently had greater effect on the pigmentation than did light. The effect of oxygen tension on pigment production in *Rhodopseudomonas spheroides* has recently been emphasized (5).

A locally isolated strain of *Rhodopseudomonas palustris* was used in a comparative study of dehydrogenase levels. Qualitatively, the presence of the dehydrogenases was similar to that of *R. rubrum*, but there was a quantitative difference between the extracts from cells grown aerobically in the light and anaerobically in the light. The anaerobically grown cells had a greater level of dehydrogenase activity, and the citric dehydrogenase was always more active than the succinic dehydrogenase. The aerobically grown cells yielded extracts in which the activity with succinate was always highest.

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THE ENZYMES OF THE TRICARBOXYLIC ACID CYCLE OF PSEUDOMONAS AERUGINOSA[1]

By Jack J. R. Campbell and Roberts A. Smith[2]

Abstract

It was demonstrated that *Pseudomonas aeruginosa* possesses all the enzymes necessary for the oxidation of pyruvate to CO_2 and water without passing through the conventional intermediates oxalosuccinate and α-ketoglutarate. These intermediates are bypassed by the action of the enzyme isocitratase which splits *d-iso*citrate to succinate plus glyoxylate. This reaction was shown to be readily reversible. The malic acid dehydrogenase content was low and in addition this enzyme required a high pH for optimum activity. In fresh cell extracts at pH 7.4 its activity was only 10% that of the other enzymes of the cycle. The malic and *iso*citric dehydrogenases were TPN specific. The organism was also shown to possess all the enzymes necessary for the operation of the conventional tricarboxylic acid cycle.

Earlier studies with dried cell preparations of *Pseudomonas aeruginosa* revealed that this organism possessed the enzymes necessary for the oxidation of the compounds of the tricarboxylic acid cycle (3). These data were interpreted as indicating that a conventional cycle was present. On more detailed examination of the individual steps of the cycle it was found that the usual arsenite block, by which α-ketoglutarate accumulates during tricarboxylic acid oxidation, was bypassed. Further examination showed a rapid anaerobic dissimilation of citrate and *cis*-aconitate to glyoxylate plus succinate (2). The substrate for this reaction has now been shown to be *d-iso*citrate and the enzyme catalyzing the reaction has been named "isocitratase" (16, 17). The present study confirms the presence of the enzymes of the conventional tricarboxylic acid cycle and in addition demonstrates the sequential nature of the reactions of the alternate cycle which bypasses the steps through oxalosuccinate and α-ketoglutarate.

Methods

The organism used in these studies was *Pseudomonas aeruginosa* ATCC 9027. This is an obligate aerobe which oxidizes glucose by way of gluconic and 2-ketogluconic acids, has no hexokinase, and only a trace, if any, of aldolase. Cultures were grown in shake culture at pH 7.2 in a medium containing 0.6% sodium acetate; 0.3% ammonium dihydrogen phosphate; 0.3% dipotassium phosphate; 0.1% magnesium sulphate heptahydrate; and 5.0 p.p.m. iron added as ferrous sulphate heptahydrate. The cells were harvested at 20–24 hr., washed with *M*/30, pH 7.0, phosphate buffer, and finally suspended at a concentration of 200 mgm. wet weight of cells per ml. in 0.2% KCl solution. Cell extracts were prepared by placing this heavy suspension in the Raytheon 10 kc. oscillator for 15 min. After this treatment

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any remaining cells or large particles were centrifuged off at 28,000 *g* for 20 min. With the exception of the assay for succinic dehydrogenase, cell extracts were used throughout.

The *cis*-aconitate was prepared from the *trans* isomer by the procedure of Malachowski *et al.* (9). Sodium glyoxylate was the product of Bios Laboratories. Diphosphopyridine nucleotide (DPN), triphosphopyridine nucleotide (TPN), and the reduced form of triphosphopyridine nucleotide (TPNH) were purchased from Sigma Chemical Company. Adenosine triphosphate (ATP) was the product of Schwarz Laboratories. *Iso*citric acid, purchased as the lactone, was the product of the California Research Foundation.

Succinic dehydrogenase was determined by measuring the rate of methylene blue reduction (1/40,000 final concentration) in a Thunberg tube in the presence of 0.003 *M* cyanide at pH 7.4. Fumarase was demonstrated by measuring the increase in optical density at 240 mμ with malate as substrate (14). Each cuvette contained 0.1 ml. *M*/1, pH 7.4 tris (hydroxymethyl) amino methane (Tris) buffer; 0.01 ml. enzyme (14.2 mgm. protein/ml.); 0.79 ml. water. The reaction was started by the addition of 0.1 ml. (2.5 μM.) of malate. Malic dehydrogenase and *iso*citric dehydrogenase were determined spectrophotometrically by following the reduction of TPN at 340 mμ. For malic dehydrogenase each cuvette contained 0.1 ml. pH 7.4 or pH 8.6 Tris buffer; 0.01 ml. enzyme (14.2 mgm. protein/ml.); 0.76 ml. water; 0.03 ml. *M*/200 TPN. The reaction was started by the addition of 0.1 ml. (2.5 μM.) of malate.

For *iso*citric dehydrogenase each cuvette contained 0.1 ml. *M*/1, pH 7.4 Tris buffer; 0.01 ml. enzyme (14.2 mgm. protein/ml.); 0.01 ml. *M*/10 $MgCl_2$; 0.10 ml. (2.5 μM.) *d-iso*citrate; 0.75 ml. water. The reaction was started by the addition of 0.03 ml. *M*/200 TPN.

Reversal of malic acid oxidation was investigated by following TPNH oxidation at 340 mμ on the addition of oxalacetate or of pyruvate plus bicarbonate. ATP (1 μM.) was also added in an effort to promote this reaction. Condensing enzyme was measured by determining citrate accumulation with pyruvate as substrate (7). Each Warburg cup contained 1.5 ml. *M*/15, pH 7.0 phosphate buffer; 1.0 ml. cell extract (14.2 mgm. protein/ml.); 0.2 ml. (5 μM.) pyruvate, water to 3.0 ml. One vessel was removed at each 20 min. interval and analyzed for citrate. Aconitase was demonstrated by using *cis*-aconitate as substrate and measuring the formation of citrate and *iso*citrate. For the *iso*citrate determination the following solutions were mixed and placed in an ice bath for 60 min.; cell extract 0.5 ml.; *M*/5, pH 7.4, glycylglycine buffer, 0.4 ml.; cysteine (10 μM./ml.) 0.2 ml. After the one hour incubation in ice the tubes were brought to 30° and 5 μM. (0.2 ml.) of *cis*-aconitate was added. Incubation at 30° continued for 10 min. The reaction was stopped by the addition of 0.2 ml. of 0.2 *M*, pH 4.0 acetate buffer and placing the mixture in a boiling water bath for four minutes. After it was cooled, the sample was centrifuged and an aliquot of the

supernatant neutralized with 1.5 *N* KOH. This aliquot could then be assayed for its *iso*citrate content by measuring TPN reduction at 340 mμ in the presence of excess *iso*citric dehydrogenase (12). The pig heart *iso*citric dehydrogenase was suspended in Tris buffer in order to minimize the formation of manganese complexes which interfere with the spectrophotometric measurement. The incubation conditions required for the formation of citrate were similar to those described above except that twice as much enzyme was used and the reaction was stopped by the addition of 0.25 ml. of 100% trichloracetic acid. In order to detect succinic acid as a product of tricarboxylic acid degradation, Warburg vessels containing 10 μM. of citrate, 1.0 ml. of cell extract, and buffer at pH 7.4 were assembled for anaerobic conditions. The reaction was allowed to proceed for 60 min.

Succinic acid was determined after ether extraction of the deproteinized reaction mixture by the manometric technique of Krebs (8). Acid–base chromatography for the detection of organic acids was according to the method of Smith (15). Butanol saturated with 4 *N* formic acid was the solvent and after development the formic acid was removed by placing the paper in a steam cabinet for 30 min. The papers were dried and then sprayed with an aqueous solution of chlorophenol red (0.04% at pH 7.0). Paper chromatography of amino acids was achieved by the method of Consden, Gordon, and Martin (6) using butanol–acetic as the solvent system. For the determination of glyoxylate the reaction mixture contained 25 μM. of substrate, 5 ml. of cell extract, 7.5 ml. of *M*/15, pH 7.4 phosphate buffer, and water to 15.0 ml. After the reaction had proceeded for 80 min. sulphuric acid was added to bring the pH to about 3.8 and the mixture was held at 60° for five minutes to precipitate the protein. The precipitate was removed by centrifugation and the glyoxalate was determined on the supernatant as the 2,4-dinitrophenylhydrazone after the method of Cavallini *et al.* (4).

Experimental

Cell extracts of *Pseudomonas aeruginosa* oxidized citric, *cis*-aconitic, *iso*citric, α-ketoglutaric, succinic, fumaric, malic, and oxalacetic acids thus satisfying one criterion for the presence of a conventional tricarboxylic acid cycle. However, these data do not demonstrate that a cyclic mechanism is involved or that the compounds are oxidized sequentially. Evidence for the presence of one sequence of reactions of the conventional cycle was obtained by the use of an aged cell extract which has lost its ability to oxidize oxalacetic acid. As indicated by oxygen uptake studies, the cell extract was found to oxidize the 5 μM. of succinate, fumarate, or malate quantitatively to oxalacetate (Table I).

It would appear, therefore, that succinate, fumarate, and malate are oxidized by way of oxalacetate and thus this part of the cycle is identical with the comparable steps in the Krebs cycle.

The presence of succinic dehydrogenase was confirmed by the Thunberg technique. Although the rate of endogenous respiration of cells harvested at

TABLE I

OXIDATION OF DICARBOXYLIC ACIDS TO OXALACETATE

Substrate	Oxygen uptake (atoms/μM. substrate)	
	Actual	Theoretical
Succinate	2.00	2.00
Fumarate	0.98	1.00
Malate	1.00	1.00
Oxalacetate	0.00	0.00

18–24 hr. is not sufficient to interfere with oxygen uptake studies, nevertheless these cells were of no value for studies involving a hydrogen acceptor such as methylene blue. It was necessary to harvest cells at 9–10 hr. in order to ensure that the rate of endogenous respiration was sufficiently low to permit the measurement of substrate dehydrogenation. Using the 9–10 hr. cells it was found that, in the presence of succinate and cyanide, methylene blue was reduced within 20 min. while the endogenous control remained unchanged for at least three hours. Cyanide did not influence the rate of reduction.

The sonic extract of *P. aeruginosa* was shown to contain a high concentration of the enzyme fumarase (Fig. 1). Accepting an extinction coefficient of 2.11×10^6 (cm.^{2} $\times$ moles^{-1}) for fumarate at 240 mμ it can be calculated that fumarate was produced at the rate of 0.45 μM./mgm. protein/min.

The malic dehydrogenase was found to be present in relatively small amounts and in addition the optimum activity of this enzyme was found to be at pH 8.6 (Fig. 3). When allowed to react at this pH, malate was dehydrogenated at the rate of 0.193 μM./mgm. protein/min. At the more physiological pH of 7.4 the activity was reduced to 0.045 μM./mgm. protein/min. One minor idiosyncrasy of the enzyme is that it is TPN specific. The reduction of TPN in the presence of malate, therefore, could be accomplished either by malic dehydrogenase or by Ochoa's "malic enzyme". In order to determine which of these two was active, efforts were made to oxidize TPNH by the addition of the respective end products. No reversal was achieved on the addition of pyruvate plus bicarbonate whereas slow reversal was evident on the addition of oxalacetate. This indicates that the oxidation of malate was carried out by a TPN linked malic dehydrogenase.

The presence of the condensing enzyme was determined by analyzing for the formation of citrate during pyruvate oxidation (Fig. 2). This pronounced formation of citrate during the oxidation of pyruvate indicates either a very strong "C_1" fixation mechanism or the presence of appreciable quantities of dicarboxylic acids in the cell extracts. These acids could not be detected either by acid–base paper chromatography or by chromatographic analysis for amino acids. Moreover, the addition of succinate greatly increased citrate accumulation. It is therefore proposed that the added pyruvate is the source of both of the substrates required for the condensing reaction.

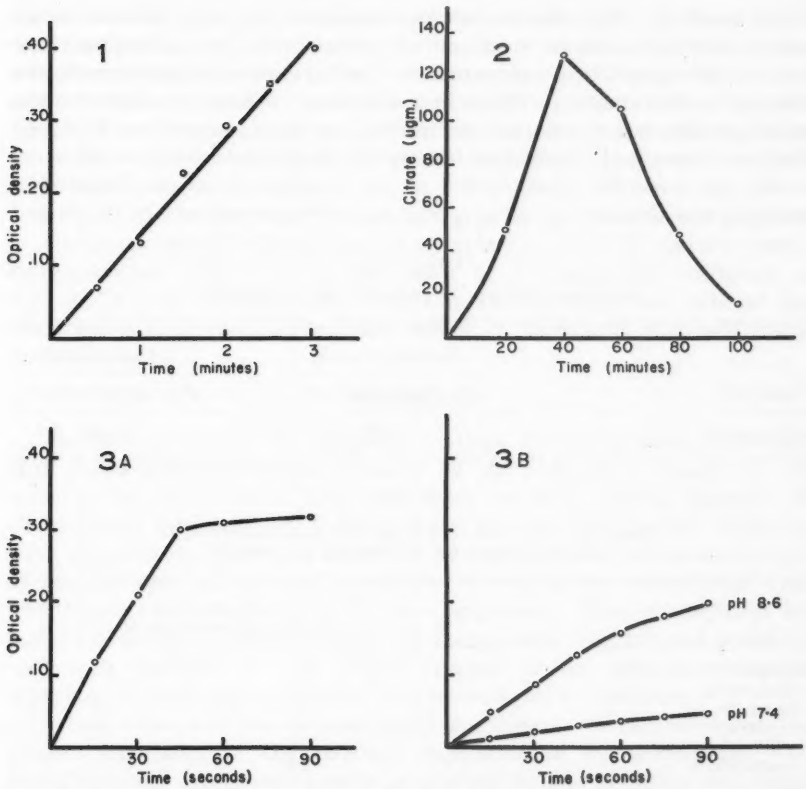

FIG. 1. Fumarase activity in extracts of *P. aeruginosa.*
FIG. 2. Condensing enzyme in extracts of *P. aeruginosa.*
FIG. 3A. *Iso*citric dehydrogenase activity in extracts of *P. aeruginosa.*
FIG. 3B. Malic dehydrogenase activity in extracts of *P. aeruginosa.*

This would be comparable to the situation described by Cleland and Johnson for *Aspergillus niger* (5). On the assumption that, throughout the entire experiment, citrate was being utilized at the rate observed between the 60 and 80 min. determinations it can be calculated that essentially 100% of the pyruvate was converted to citrate. Following the same reasoning it was calculated that the condensing enzyme formed 0.35 μM. citrate/mgm. protein/min.

The presence of aconitase was shown by determining the formation of citrate and *iso*citrate from *cis*-aconitate (Table II). The rate appears to be very slow but it should be remembered that the products from the reaction are being continuously removed and it is only the presence of the aconitase and not the apparent rate that is of significance.

Efforts to detect α-ketoglutarate as a product of the oxidation of the tricarboxylic acids failed. In addition arsenite did not cause accumulation

of this product. This observation was explained by the discovery of an arsenite insensitive enzyme which splits *iso*citrate to succinate plus glyoxylate. In an earlier report (2) it was stated that *iso*citrate did not appear to be the substrate of this enzyme. This was found to be an error due either to the use of grossly impure samples of *iso*citrate or to its conversion to *trans*-aconitate under acid conditions. Glyoxylate was recovered from both the aerobic and anaerobic dissimilation of the tricarboxylic acids (Table III). Succinate was also determined as a product of these reactions and Smith and

TABLE II

ACONITASE ACTIVITY IN EXTRACTS OF *P. aeruginosa*

Substrate	*Iso*citrate formed μM./mgm.protein/hr.	Citrate formed μM./mgm.protein/hr.
cis-Aconitate	0.075	0.084

TABLE III

FORMATION OF GLYOXYLATE BY *P. aeruginosa* DURING DEGRADATION OF TRICARBOXYLIC ACIDS

	R_F values of 2,4-dinitrophenylhydrazones*	
Standards		
Oxalacetate	.31	
Pyruvate	.50	.65
Glyoxylate	.41	.57
α-Ketoglutarate	.35	
Acetaldehyde	.90	
Reactions		
Citrate oxidation	.42	.57
cis-Aconitate oxidation	.41	.57
*Iso*citrate oxidation	.41	.57
Citrate fermentation	.43	.56
cis-Aconitate fermentation	.42	.57
*Iso*citrate fermentation	.41	.57

* *Solvent: n-butanol 50, ethanol (95%) 10, water 40.*

TABLE IV

REVERSIBILITY OF ISOCITRATASE REACTION

Substrates	Citrate formed at 100 min., μM.
2.5 μM. succinate + 2.5 μM. glyoxylate	.59
2.5 μM. glyoxylate	.03
2.5 μM. succinate	.06
2.5 μM. fumarate + 2.5 μM. glyoxylate	.05
2.5 μM. fumarate	.06
Endogenous	.04

Gunsalus (17) have now demonstrated the stoichiometry of the reaction catalyzed by the enzyme "isocitratase". The reversibility of this reaction was demonstrated by incubating succinate plus glyoxylate with the cell extract under anaerobic conditions and analyzing for citrate formation (Table IV). These experiments indicate that the reaction is readily reversible. It is also evident that citrate is not being formed by the action of the condensing enzyme.

In spite of the fact that it was not possible to detect α-ketoglutarate accumulation the cell extracts do contain a very active TPN specific *iso*citric dehydrogenase (Fig. 3). On the basis of an extinction coefficient of 6.22×10^6 (cm.^{2} $\times$ mole^{-1}) for TPNH at 340 mμ these data indicate that *iso*citrate is being oxidized by this system at the rate of 0.40 μM./mgm. protein/min.

Discussion

The demonstration of the foregoing reactions makes it possible to conclude that *Pseudomonas aeruginosa* contains all the enzymes necessary for the oxidation of pyruvate to CO_2 and water, without passing through the conventional intermediates oxalosuccinate and α-ketoglutarate. However, since the presence of a strong *iso*citric dehydrogenase was demonstrated, some of the pyruvate undoubtedly is oxidized by way of the conventional cycle and therefore is converted to these two compounds. This new pathway has now been shown to occur in many microorganisms (13, 18) and makes an interesting addition to our present concept of the importance of the tricarboxylic acid cycle as a source of intermediates for synthesis.

Certain aspects of the present study are difficult to reconcile with our present knowledge of the terminal respiration of microorganisms. One would normally expect succinate to be oxidized by way of malate and indeed in the case of the aged extracts used here this appeared to be the only route available. However, in fresh extracts the malic dehydrogenase was relatively weak even at its optimum pH, which is approximately 8.6. At pH 7.4 the activity of the enzyme was extremely low and would suggest that one should be able to detect the accumulation of malate. No malate was evident on acid–base paper chromatograms and it is possible that fresh extracts have an alternate system for the degradation of dicarboxylic acids. It was not possible to present a complete quantitative picture on the enzymes of the cycle because the products of many of the reactions were being continuously removed and in addition several enzymes are competing for a substrate such as *iso*citrate.

An additional idiosyncrasy of this organism is that it evidently contains the enzyme "aconitic hydrase" (1), previously reported by Neilson (10, 11) to be present in extracts of *Aspergillus niger*.

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THE EFFECT OF TRACE ELEMENTS ON GROWTH OF HELMINTHOSPORIUM SATIVUM AND SEVERAL RELATED SPECIES[1]

By E. A. Peterson and H. Katznelson

Abstract

Zinc, manganese, and iron were shown to be essential for good growth of *Helminthosporium sativum*, whereas copper, cobalt, molybdenum, boron, and calcium apparently were not required. One milligram each of zinc sulphate, manganese chloride, and ferrous sulphate per liter was considered adequate to ensure maximum growth of *H. sativum* in simple media. *H. biforme*, *H. halodes*, and *H. setariae* also produced maximum growth in response to a combination of zinc, manganese, and iron.

Introduction

Previous studies at this laboratory (4) have shown that *Helminthosporium sativum* requires an adequate supply of trace elements for good growth in simple media. Zinc was found to exert the greatest influence on yield of mycelium; the results indicated in addition that manganese, iron, and boron may also be important in the nutrition of the fungus. On the basis of these results, a series of tests was conducted in which the experimental media were treated to reduce trace element contamination before supplementing with known trace elements. The results of these tests are reported in this paper.

Methods

The composition of the basal medium in grams per liter of distilled water was: sucrose, 30; KH_2PO_4, 1.0; K_2HPO_4, 1.3; KCl, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5. For comparison, sodium nitrate at 2.0 gm. per liter or *l*-proline at 2.7 gm. per liter was used as the nitrogen source.

Except where stated otherwise, the nitrogen supplemented basal medium was treated with chromatographic Al_2O_3 to reduce trace element contamination according to the procedure (Method 5) described by Donald, Passey, and Swaby (1). Salts of trace elements, prepared as stock solutions with double-distilled water, were added to the treated medium to give the following concentrations in milligrams per liter: $FeSO_4 \cdot 7H_2O$, 10.0; $ZnSO_4 \cdot 7H_2O$, 2.0; $MnCl_2 \cdot 4H_2O$, $CuSO_4 \cdot 5H_2O$, $CoCl_2 \cdot 6H_2O$, each 0.4; $Na_2MoO_4 \cdot 2H_2O$, 0.25; H_3BO_3, $CaCl_2$, each 0.6. The reaction of all media was adjusted to pH 6.0 before sterilization.

Conditions of cultivation were similar to those described previously (4). Stock cultures were maintained on potato dextrose agar slants. Inoculum consisted of a suspension of washed spores and mycelial fragments prepared with double-distilled water from an agar culture 10 to 15 days old. The suspension was standardized to contain approximately 1000 spores and

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Contribution No. 405 from the Bacteriology Division, Science Service, Canada Department of Agriculture, Ottawa, Canada.

fragments per milliliter. It was understood of course that trace elements could be carried over with this inoculum. However, owing to the difficulty experienced in producing a suitable crop of spores in an inorganic medium and since material from even such a medium would also contain trace elements, it was considered that the inoculum as prepared was adequate for the present purpose. Test cultures were grown for 20 days, except where stated otherwise, at 26° C. in 125 ml. Erlenmeyer flasks fitted with 50 ml. beakers in place of cotton plugs. Each flask contained 25 ml. of medium and received 1 ml. of inoculum. Mycelial mats were collected by means of a 200-mesh copper wire screen, washed, dried overnight at 85° C., and weighed. All glassware was washed with Alconox solution and rinsed thoroughly with tap water, followed by autoclaving and rinsing in distilled and double-distilled water.

Results

Effect of Trace Elements on Growth Rate

Fig. 1 illustrates the influence of a mixture of trace elements on the growth of *H. sativum* in the basal medium with the nitrogen sources *l*-proline or sodium nitrate at an equivalent nitrogen level. Although the two media were not treated to reduce trace element contamination in this experiment, a striking response was obtained on the addition of trace elements. On prolonged incubation abundant mycelial growth occurred with both nitrogen compounds. However, the fungus grew faster and maximum growth resulted earlier in the presence of amino nitrogen.

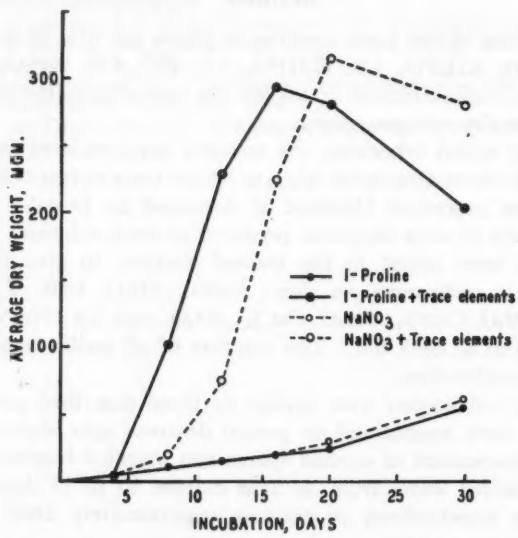


FIG. 1. The influence of trace elements and nitrogen sources on the growth rate of *H. sativum* in liquid media. Plotted points each represent the average of four determinations.

Trace Element Deficiencies

The effect of individual trace element deficiencies on the growth of *H. sativum* is shown in Table I. A deficiency of zinc, manganese, or iron in the otherwise complete medium resulted in a marked reduction in mycelial yield. No such effect was apparent with corresponding deficiencies of copper, cobalt, molybdenum, boron, or calcium. Deficiencies of zinc, manganese, and iron were most evident in the sodium nitrate medium. With the exception of zinc, the deficiencies were not nearly so marked in the *l*-proline medium; however, trace elements are generally considered to be difficult to remove from media containing amino acids because of their chelating properties. It would appear that complete removal of trace elements has not been achieved by the Al_2O_3 treatment. Nevertheless some purification has been accomplished since the response to the addition of manganese, for example, was markedly greater in the purified (Table I) than in the unpurified sodium nitrate medium as shown in Table V of a previous paper (4).

TABLE I

EFFECT OF TRACE ELEMENT DEFICIENCIES ON GROWTH OF *Helminthosporium sativum*

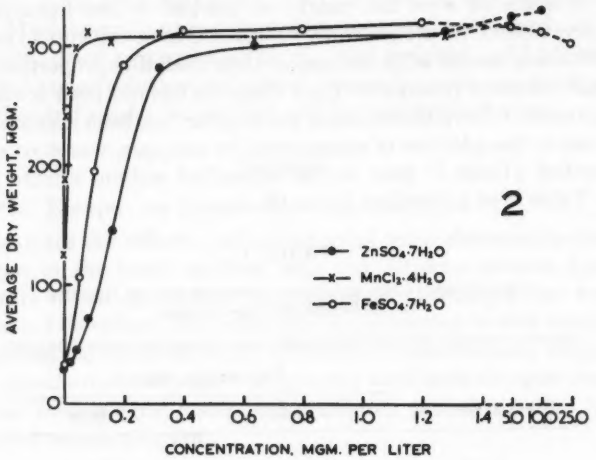
Element omitted	Dry weight, mgm.*	
	$NaNO_3$	*l*-Proline
None	76.2	258.2
Fe	30.5	166.4
Zn	12.6	8.9
Mn	11.3	212.1
Cu	70.5	274.3
Co	71.1	262.9
Mo	88.8	271.7
B	70.4	255.7
Ca	63.5	272.7
All	14.4	25.0

**Each weight is the average of three determinations at 14 days' incubation.*

Relation of Concentration of Zn, Mn, and Fe to Growth

Since zinc, manganese, and iron were considered to be essential for good growth of *H. sativum*, it was of interest to obtain some indication of their optimum concentrations. Accordingly, a series of Al_2O_3-treated media was prepared in which increasing amounts of zinc, manganese, and iron were tested. While the concentration of any one of the three elements was varied, the remaining two were included at the fixed amounts mentioned earlier. Figs. 2 and 3 show a marked similarity in the growth response of *H. sativum* to increasing amounts of zinc, manganese, and iron with the two nitrogen sources, *l*-proline and sodium nitrate, at an equivalent nitrogen level. Satisfactory growth was obtained with as little as 0.1 mgm. of manganese chloride,

0.4 mgm. each of zinc sulphate and ferrous sulphate per liter. Maximum amounts of manganese chloride, zinc sulphate, and ferrous sulphate tested were 5.0, 10.0, and 25.0 mgm. per liter respectively, no adverse effects on growth being evident at these levels. On the basis of these results, 1.0 mgm. per liter of each of the above-mentioned trace element-containing compounds was considered adequate to ensure maximum growth of *H. sativum*.



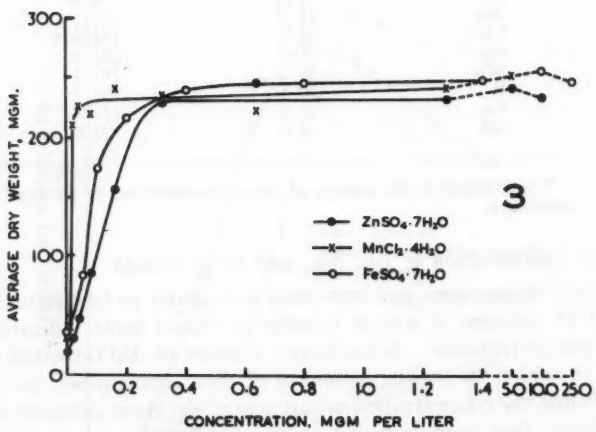


FIG. 2. The effect of different concentrations of zinc, manganese, and iron on growth of *H. sativum* with *l*-proline as nitrogen source.

FIG. 3. The effect of different concentrations of zinc, manganese, and iron on growth of *H. sativum* with sodium nitrate as nitrogen source.

TABLE II

GROWTH RESPONSE OF *Helminthosporium sativum* AND SEVERAL RELATED SPECIES TO COMBINATIONS OF TRACE ELEMENTS

Nitrogen source	Trace element supplements	Dry weight of mycelium in mgm.*			
		H. sativum	*H. biforme*	*H. halodes*	*H. setariae*
$NaNO_3$	All	255.1 (5.6)	125.0 (6.0)	292.5 (0.5)	267.8 (0.8)
$NaNO_3$	Zn, Mn, Fe	258.4 (3.2)	231.0 (13.0)	282.3 (3.7)	256.5 (1.5)
$NaNO_3$	Cu, Co, Mo, B, Ca	14.5 (3.9)	19.0 (3.0)	42.8 (5.8)	9.5 (2.0)
$NaNO_3$	None	25.8 (2.2)	16.3 (4.8)	54.8 (10.3)	26.5 (2.5)
l-Proline	All	246.4 (2.4)	327.9 (1.1)	345.7 (0.7)	307.5 (3.0)
l-Proline	Zn, Mn, Fe	262.2 (4.7)	333.0 (2.0)	341.7 (1.3)	310.5 (3.0)
l-Proline	Cu, Co, Mo, B, Ca	23.2 (0.3)	64.5 (0.0)	204.3 (4.3)	134.3 (1.8)
l-Proline	None	26.8 (0.1)	87.3 (0.3)	195.8 (6.7)	81.5 (7.5)

**Each weight is the average of two determinations with the range given in brackets. Incubation time was 20 days.*

Species Response to Combinations of Trace Elements

For comparative purposes, *H. sativum* and several related species were grown in Al_2O_3-treated media which had been supplemented with the trace element combinations: (*a*) zinc, manganese, iron and (*b*) copper, cobalt, molybdenum, boron, calcium. The complete medium, including the eight elements, and the unsupplemented medium served as controls. Table II shows that all species tested responded very favorably to the presence of zinc, manganese, and iron with either *l*-proline or sodium nitrate. No such effect was obtained with the remaining elements. In the nitrate series *H. biforme* appears to be sensitive to some constituent in the complete medium which was omitted in the zinc, manganese, iron combination. The effect was not apparent in the proline series, possibly because of a sequestering effect by this amino acid.

Discussion

It has been demonstrated that zinc, manganese, and iron are essential for good growth of *Helminthosporium sativum*. *H. biforme*, *H. halodes*, and *H. setariae* also responded favorably to a combination of these elements. Although copper, cobalt, molybdenum, boron, and calcium had little, if any, influence on growth, it is not possible to classify them as non-essential, since trace elements were still present in the purified media. Despite the precautions taken to reduce trace element contamination in the media used, there were at least two additional sources of contamination, namely, the spore inoculum and the trace element solutions, both of which were added after the media were purified. Spores are known to carry trace elements and may even store them (2). The compounds used to prepare the trace element solutions

usually contain various elements as impurities. Consequently, sufficient quantities of other elements which may be required only in extremely minute amounts may well be supplied in the form of impurities.

By the use of more efficient purification techniques, or by the use of a chelating agent such as ethylenediamine – tetraacetic acid as discussed by Hutner *et al.* (3), it may be possible to demonstrate requirements for other elements by *H. sativum*.

Acknowledgment

The authors are grateful to Miss Margaret I. Chisholm for able technical assistance.

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GLUCOSE OXIDATION BY SERRATIA MARCESCENS[1]

By Aaron E. Wasserman, William Hopkins, and Thomas S. Seibles

Abstract

Fresh cell suspensions of glucose-grown *Serratia marcescens* oxidize glucose, gluconic acid, and 2-ketogluconate with an uptake of 3.0, 2.5, and 2.0 μmoles of oxygen per μM. substrate, respectively. Approximately two μM. of CO_2 per μM. of each substrate are evolved. 6-Phosphogluconate, glucose-6-phosphate, and hexose diphosphate do not appear to be intermediates in the normal pathway of glucose oxidation. Dried-cell preparations of *S. marcescens* oxidize glucose and gluconate in a two-stage pattern, with an initial uptake of 1.0 and 0.5 μM. O_2/μM. substrate, respectively. 2-Ketogluconate was identified chromatographically in these solutions. In the second stage, both added and metabolically formed 2-ketogluconate are further oxidized to the same oxidation level attained with the whole cell, i.e., 3.0, 2.5, and 2.0 μM. O_2/μM. glucose, gluconate, and 2-ketogluconate, respectively. An as yet unidentified acidic compound has been isolated from the solution of metabolites of a glucose oxidation by these organisms.

Introduction

In the first systematic investigation of glucose dissimilation by *Serratia marcescens* it was found that lactic acid was produced in large quantities. Substantial amounts of acetoin and 2,3-butylene glycol, and traces of ethanol, acetic acid, and formic acid were also formed (8). One strain of *Serratia marcescens* formed approximately 12% succinic acid. Further investigation of glucose metabolism (5), indicated that approximately 50% of the glucose went to form 2,3-butylene glycol. The remainder gave rise to lactic acid and ethanol, with traces of glycerol and succinic acid also being found. The production of hydrogen anaerobically led to a scheme for the division of the genus *Serratia* into three groups. In two of these groups 2,3-butylene glycol is the major end product, and in the third group glucose is dissimilated primarily to acetic acid (4, 6).

The metabolic pathways by which *S. marcescens* dissimilates glucose have not been investigated. However, with a cell-free extract of a 2-ketogluconate adapted strain of *S. plymouthicum* it has been shown that 2-ketogluconate was phosphorylated to 2-keto 6-phosphogluconate (2).

The studies reported here indicate that cells of glucose-grown *S. marcescens* dissimilate glucose via gluconic and 2-ketogluconic acids to an acidic end product which has not yet been identified.

Materials and Methods

A laboratory strain of *S. marcescens*, obtained from the Northern Utilization Research Branch, U.S. Department of Agriculture, was grown in a glucose–peptone–milk protein medium on a reciprocal shaker at 37° for 17 hr. Cell suspensions in water were adjusted to the required dry weight, using a

[1]*Manuscript received February 7, 1956.*
Contribution from the Eastern Regional Research Laboratory, Philadelphia 18, Pennsylvania, U.S.A. (A Laboratory of the Eastern Utilization Research Branch, Agricultural Research Service, United States Department of Agriculture.)

precalibrated dry weight vs. turbidity curve prepared with a Beckman model B spectrophotometer. Freeze-dried preparations of the organism were obtained by freezing a thick cell suspension at −70°, and vacuum drying with a Hy-Vac pump.

Warburg determinations were carried out by conventional manometric techniques. Gas exchange values are reported as μM. of gas (O_2 or CO_2) per μM. of substrate. The data reported are the average values of three to six experiments each.

Chromatographic analysis of glucose, gluconate, and 2-ketogluconate was carried out by modification of the methods of Partridge (7) and White (11). The paper chromatograms were developed by ascending technique in methanol : ethanol : water (45 : 45 : 10) solvent. After drying, the paper was sprayed with triphenyltetrazolium chloride reagent (50 mgm. triphenyltetrazolium chloride dissolved in a mixture of: 5% sodium hydroxide, 10 ml.; *n*-butanol, 30 ml.; ethanol, 20 ml.). Color was developed by steaming the paper. Glucose and 2-ketogluconate appeared as cherry-red spots. Gluconate did not react with the reagent. After drying, the paper was sprayed with silver nitrate reagent (0.1 *N* silver nitrate in 5 *N* ammonium hydroxide). When the paper was free of ammonia, steam was again applied. Gluconate, with an R_f similar to that of 2-ketogluconate, appeared as a dark brown spot.

Acidic compounds were determined chromatographically by the method of Martin (3). Chromatographic analysis for phosphate esters was performed by the method of Bandurski and Axelrod (1). Quantitative colorimetric analyses were carried out by the methods of Somogyi (9) for glucose, Westerfeld (10) for acetone, and Winnick (12) for 2,3-butylene glycol.

Materials

Gluconolactone, from Eastern Chemical Corporation[2] was the source of gluconic acid used in these studies. 6-Phosphogluconate was obtained from the Delta Chemical Works, Inc. 2-Ketogluconate was supplied by the Northern Utilization Research Branch, Peoria, Illinois, through the courtesy of Dr. F. H. Stodola. Glucose-6-phosphate was obtained from the Nutritional Biochemical Corp. Hexose diphosphate was obtained from Schwarz Laboratories.

Results

The oxidative behavior of one preparation of a fresh cell suspension of *S. marcescens* on glucose and several possible intermediates is shown in Fig. 1. Glucose, gluconate, and 2-ketogluconate were oxidized with an uptake of approximately 3.0, 2.5, and 2.0 μM. O_2 respectively. Glucose-6-phosphate and 6-phosphogluconate were oxidized to a level of 1.0–1.5 μM. O_2 depending upon the cell preparation, while hexose diphosphate was metabolized to a level of about 0.7 μM. O_2. Table I shows CO_2 evolution from these substrates, and O_2 uptake values obtained simultaneously. Approximately 2 μM. of

[2]*Mention of trade names or companies does not imply recommendation by the United States Department of Agriculture over similar products not mentioned.*

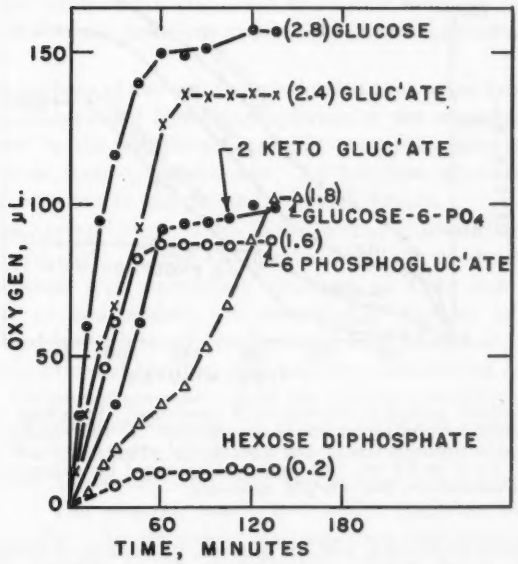

FIG. 1. Metabolic pattern of fresh *S. marcescens*. Each flask contains 0.5 ml, 0.05 *M* Tris (hydroxymethyl) amino methane buffer, pH 7.2; 100 γ inorganic phosphorus; 0.5 ml. cell suspension (containing 4 mgm. dry weight); 2.5 μM. of appropriate carbohydrate; water to 2.0 ml. All values are corrected for endogenous metabolism. Numbers in parenthesis are μM. O_2/μM. substrate.

TABLE I

GAS EXCHANGE DURING OXIDATION BY *S. marcescens*

	O_2*	CO_2*
Glucose	3.0	2.4
Gluconate	2.2	2.2
2-Ketogluconate	2.0	1.9
6-Phosphogluconate	1.3	2.1
Glucose-6-phosphate	1.0	1.5
Hexose diphosphate	0..7	0.8

*μM. gas/μM. substrate.

Each flask contains 0.5 ml. 0.05 M Tris (hydroxymethyl) amino methane buffer, pH 7.2; 100 γ inorganic phosphorus; 0.5 ml. cell suspension (containing 4 mgm. dry weight); 2.5 μM. of appropriate substrate; water to 2.0 ml. Values are corrected for endogenous metabolism. These values are the average of three to six experiments with several cell preparations.

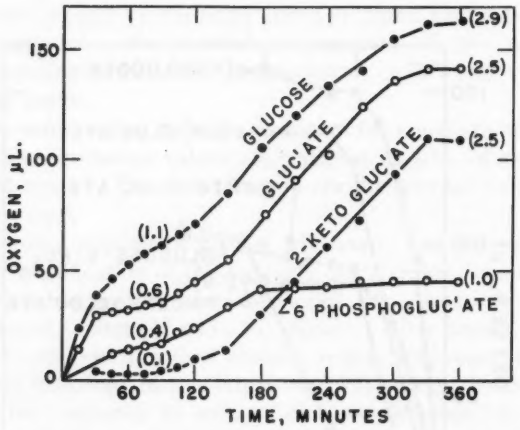

FIG. 2. Metabolic pattern of freeze-dried *S. marcescens*. Each flask contains 0.5 ml. 0.05 *M* Tris (hydroxymethyl) amino methane buffer, pH 7.2; 100 γ inorganic phosphorus; 0.5 ml. dried-cell suspension (containing 8 mgm. dry weight); 2.5 μM. of appropriate carbohydrate; water to 2.0 ml. All values are corrected for endogenous metabolism. Numbers in parenthesis are μM. O_2/μM. substrate.

CO_2 were formed from each μM. of glucose, gluconate, 2-ketogluconate, and 6-phosphogluconate. Approximately 1 μM. of CO_2 was obtained from glucose-6-phosphate and hexose diphosphate.

In an attempt to determine whether permeability was a factor in metabolic behavior of the phosphorylated intermediates, freeze-dried cells of *S. marcescens* were employed in a similar series of experiments. As shown in Fig. 2, the rates of oxidation of all substrates by the dried cells were generally lower than that of fresh cells. The oxidation of glucose and gluconate occurred in a two-stage pattern. First, glucose and gluconate were metabolized with an uptake of 1.0 and 0.5 μM. O_2 respectively. 2-Ketogluconate was oxidized slowly, if at all. In the second stage, the rate of 2-ketogluconate oxidation increased, and the oxidation of glucose and gluconate were resumed. The rate of oxidation of glucose and gluconate paralleled that of the 2-ketogluconate oxidation, leading to the supposition that the same material was being utilized in all three instances. The final levels of oxidation for these substrates were the same as for fresh cell suspensions, namely 3.0 μM. O_2 for glucose, 2.5 μM. O_2 for gluconate, and 2.0 μM. O_2 for 2-ketogluconate. Hexose diphosphate was not oxidized.

6-Phosphogluconate was metabolized slowly to a level of 0.5 μM. O_2, then the oxidation continued to a final level of 1.0–1.5 μM. O_2.

Chromatographic analysis of the metabolite solutions at stage one revealed the presence of 2-ketogluconate in the flasks originally containing glucose and gluconate and not in the solution containing 6-phosphogluconate. Analysis of the solutions at the completion of the experiments showed the complete absence of 2-ketogluconate in the solutions in which it had accumulated.

Chromatographic analyses for phosphate esters were carried out at various times during the course of oxidation, and at no time were any phosphorylated compounds found in the solutions containing glucose, gluconate, or 2-ketogluconate.

Colorimetric analysis of the solutions indicated that when glucose was used as substrate it disappeared by the completion of the experiment. Acetoin was not formed in the solutions originally containing glucose, gluconate, 2-ketogluconate, or 6-phosphogluconate. 2,3-Butylene glycol was not found in the oxidized solutions of glucose and 2-ketogluconate.

However, the gas exchange values indicate that glucose dissimilation was accomplished with 50% of the O_2 uptake and 33% of the CO_2 evolution expected if glucose was completely oxidized to CO_2 and water. The accumulation of an end product was suspected. Glucose, in the Warburg medium described under Fig. 1, was oxidized by a suspension of fresh cells until the reaction attained the rate of endogenous metabolism after taking up 3.0 μM. O_2. Following the removal of the cells by centrifugation, and the halting of further enzymatic activity by either boiling the metabolite solution or adding 20% trichloracetic acid, the solution was run through a cation exchange column (Dowex-50) and then through an anion exchange column (Duolite A-4). The neutral, basic, and acidic fractions were examined chromatographically. In the acidic fraction there was an accumulation of material great enough to be investigated. Using larger quantities of metabolite solution, an acidic, saturated, nonreducing compound was isolated. Chemical identification of this compound has not been completed, but chromatographically it does not appear to be identical to many acidic compounds normally found as end products of glucose metabolism.

Discussion

Fresh cell suspensions of *S. marcescens*, grown on a glucose-containing medium, oxidized glucose, gluconate, and 2-ketogluconate rapidly without an initial lag period. The final values of oxygen utilization—3.0 μM. for glucose, 2.5 μM. for gluconate, and 2.0 μM. for 2-ketogluconate—were in the same relative order as the initial oxidative levels of these substrates, indicating that the same end product was formed from all three substrates. This relation was further confirmed by the evolution of approximately the same amount of CO_2, 2.0 μM. for each of the substrates. If 6-phosphogluconate were an intermediate compound in the metabolic pathway it would be expected that the amount of O_2 taken up would be the same as that for gluconic acid. However, the fact that 1 μM. less O_2 was utilized suggests that 6-phosphogluconate is oxidized via another path. Impermeability of the cell to 6-phosphogluconate may be discounted, inasmuch as the initial rate of 6-phosphogluconate oxidation was as rapid as that of the other substrates. Glucose-6-phosphate was oxidized to the same level as 6-phosphogluconate. If glucose-6-phosphate was first oxidized to 6-phosphogluconate it should show an O_2 uptake of 0.5 μM. greater than that of 6-phosphogluconate. In addition, the initial rate

of glucose-6-phosphate was slow, suddenly increasing to parallel the rates of the other substrates. This lag could be caused by the conversion of glucose-6-phosphate into a more readily oxidizable compound, or by the need for the formation of enzymes capable of utilizing the substrate itself.

The low oxidation of hexose diphosphate may be explained by the impermeability of the cell to hexose diphosphate, or by the lack of enzymes capable of converting hexose diphosphate into a more utilizable form.

The utilization of freeze-dried preparations of *S. marcescens* further confirmed the fact that glucose was oxidized via gluconate and 2-ketogluconate. The cells thus treated oxidized glucose and gluconate to 2-ketogluconate, which accumulated temporarily. During the same time period the cells were able to oxidize 2-ketogluconate very slowly, if at all. Oxidation of 2-ketogluconate then began at the same time in all three substrate solutions and continued to the same oxidation level for each substrate attained by the fresh cells. It appears, therefore, that during the freeze-drying treatment the cells are injured to such an extent that their normal metabolic pattern is interrupted. The nature of this injury is unknown. Altered permeability may allow leakage of a cofactor necessary for 2-ketogluconate oxidation when the dried cells are restored in water. The lag period would be required for the cell to rebuild the missing component. This would be true also if the enzyme responsible for this oxidation were destroyed in freezing or dehydration.

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THE REVERSAL OF QUATERNARY AMMONIUM DETERGENT INHIBITION OF SACCHAROMYCES CEREVISIAE BY A FRACTION DERIVED FROM YEAST EXTRACT[1]

By Marvin A. Rich and Arthur M. Stern

Abstract

It was demonstrated that yeast extract is capable of reversing the inhibition of *S. cerevisiae* by a quaternary ammonium surface active agent. A reversing factor representing a 25-fold increase in activity was obtained by chemical fractionation of the yeast extract, and the chemical nature of this reversing factor was partially characterized. It was observed that the growth response of cells which had been inhibited and then reversed was the same as that of uninhibited cells. Mechanisms of reversal compatible with the experimental results have been suggested.

Introduction

Although the quaternary ammonium surface active agents play an important role in medical and industrial disinfection, the fundamental principles governing their action are either unknown or in a highly controversial state. Reversibility of inhibition has been demonstrated with many antimicrobial substances including chlorine (10), mercurials (3), sulphanilamide (14), penicillin (9), and streptomycin (5). Valko and DuBois (13) reported the neutralization of cationic detergents by anionic detergents. They found, however, that the anionic detergent had to be added within 15 to 30 min. after the addition of the inhibitor. Similar cases have been reported by Baker *et al.* using phospholipids (2), Lawrence using a naphthalene sulphonic acid derivative (8), and Rahn who used various combinations of lecithin, charcoal, anionic substances, and skimmed milk (11, 12). In all reported cases, however, it was necessary to add the so-called reversing agent immediately, or within a short time after the addition of the inhibiting agent. This is indicative of an inactivation of the inhibitor, rather than a *true* reversal of inhibition. While we were studying resistance to quaternary ammonium compounds, an apparently *true* reversal of quaternary ammonium inhibition by yeast extract (Difco) was encountered. This study represents an investigation of this reversal by yeast extract and by a substance obtained through the chemical fractionation of yeast extract.

Materials and Methods

Bacteriological

The composition of the defined medium used throughout this study was as follows: sucrose, 3.4 gm.; aspartic acid, 0.17 gm.; ammonium citrate, 3.4 gm.; ammonium sulphate, 5.0 gm.; potassium phosphate (dibasic), 3.4 gm.;

[1]*Manuscript received March 12, 1956.*
Contribution from the Department of Bacteriology, Rutgers University, New Brunswick, New Jersey.
A portion of this paper was presented in part at the annual meeting of the Society of American Bacteriologists held in New York City, May 8–12, 1955.

mineral salt solution,* 10 ml.; inositol solution,† 9 ml.; thiamine solution, 10 ml.; pyridoxine solution, 10 ml.; calcium pantothenate solution, 10 ml.; casein hydrolyzate (vitamin free), 10 ml.; biotin solution, 1 ml.; distilled water to 1000 ml. The pH was adjusted to 5.3 and the medium autoclaved at 121° C. for 12 min.

The organism employed in these studies was *Saccharomyces cerevisiae*, a yeast which grows well in liquid media making it amenable to turbidimetric determinations. Preliminary studies indicated that this organism was sensitive to the quaternary ammonium compound under investigation. Stock cultures were maintained by biweekly transfer to slants of Sabouraud glucose agar.

Inocula were prepared by suspending the growth, harvested from 24 hr. slants, in sterile distilled water. Cell concentrations were adjusted so as to give a reading of 25 on the Klett–Summerson Photoelectric Colorimeter using a filter transmitting at 640–700 millimicrons. A 0.1 ml. quantity of this suspension was used to inoculate each 10 ml. of medium.

Growth, measured as turbidity, was determined in a Klett–Summerson Colorimeter. Values of uninoculated controls were subtracted from experimental readings in all cases.

Quaternary Ammonium Detergent

The quaternary ammonium compound employed was Roccal,‡ a mixture of high molecular weight alkyl-dimethyl-benzyl ammonium chlorides. A stock solution of 1 : 1000 was prepared weekly from the concentrated material. Roccal was added in varying concentrations to replicate series of tubes containing supplemented basal medium. After autoclaving, the medium was cooled, inoculated, and incubated at 28° C. for 18 hr. unless otherwise specified. After incubation, the tubes were agitated uniformly to disperse the cells and turbidities measured.

Agents Tested for Protective Action

The agents tested for protective activity were added in varying concentrations to basal medium containing Roccal. A list of the substances screened may be found in Table I. The components of yeast extract (Difco), as determined by assay,§ were tested individually and in combination (so as to simulate yeast extract) for protective activity. The cellular debris and lysate were obtained by subjecting a suspension of *S. cerevisiae* to intermittent freezing (alcohol – dry ice mixture) and thawing.

Fractionation of Yeast Extract (Difco)

Two hundred grams of ammonium sulphate were added to 500 ml. of a 50% yeast extract solution at pH 6.6 (25° C.). The precipitate formed was

*MINERAL SALT SOLUTION: *$CaCl_2$, 450 mgm.; $MgSO_4$, 450 mgm.; H_3BO_3, 1.0 mgm.; $ZnSO_4$, 1.0 mgm.; $MnCl_2$, 1.0 mgm.; $FeCl_3$, 0.5 mgm.; $CuSO_4$, 0.5 mgm.; distilled water to 10 ml.*

†VITAMIN SOLUTIONS: *thiamine, pyridoxine, calcium pantothenate, 100 µgm./ml.; inositol, 1 mgm./ml.; biotin, 7.0 µgm./ml.*

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TABLE I

SUBSTANCES SCREENED FOR PROTECTIVE ACTIVITY

Complex nutrient substances (50 mgm./ml.)

Yeast extract (Difco)	Bacto veal (Difco)
Brain heart infusion (Difco)	Bacto liver (Difco)
Malt extract (Difco)	Beef extract (Difco)

Constituents of yeast extract

Inositol, 27 μgm./ml.	L-Glutamic acid, 35 mgm./ml.
Ca pantothenate, 3 μgm./ml.	Glycine, 10 mgm./ml.
Thiamine, 3 μgm./ml.	L-Histidine, 4.5 mgm./ml.
Pyridoxine, 3 μgm./ml.	L-Isoleucine, 15 mgm./ml.
Biotin, 0.3 μgm./ml.	L-Leucine, 20 mgm./ml.
Nicotinamide, 25 and 50 μgm./ml.	L-Lysine, 20 mgm./ml.
Riboflavin, 25 and 50 μgm./ml.	DL-Methionine, 40 mgm./ml.
Niacin, 25 and 50 μgm./ml.	L-Phenylalanine, 10 mgm./ml.
B_{12}, 0.3 and 6 μgm./ml.	L-Threonine, 15 mgm./ml.
Folic acid, 1 μgm./ml.	L-Tryptophane, 4.5 mgm./ml.
Paraaminobenzoic acid, 1 μgm./ml.	DL-Tyrosine, 3 mgm./ml.
L-Arginine, 4 mgm./ml.	L-Valine, 15 mgm./ml.
DL-Aspartic acid, 25 mgm./ml.	

Other substances

Cellular lysate and debris of *S. cerevisiae*
Fractions derived from yeast extract
Synthetic yeast extract
Alpha-Lipoic acid*

Kindly supplied by L. J. Reed, University of Texas.

removed by filtration. The addition of more ammonium sulphate to the supernatant resulted in no further precipitation. The precipitate was dialyzed against running tap water for 40 hr. to remove ammonium sulphate. The sulphate-free residue was then removed from the dialysis sac and dried at 80° C. for 30 hr. (Fraction 14).

Chemical Tests on the Active Fraction (Fraction 14)

Using standard methods (6), the following chemical tests were performed on the active fraction: micro-Kjeldahl for nitrogen; Benedict's test for reducing sugars; the ninhydrin test for amino groups; the biuret test for peptides and proteins; the Millon test for phenolic groups; and the diphenylamine test for desoxyribonucleic acid.

Colorimetric Assay for Roccal

The methods employed for the determination of Roccal concentration were those described by Auerbach (1). This procedure was carried out with 1.0 ml. of a 30 p.p.m. Roccal solution to which had been added 5% yeast extract. The Roccal – yeast extract mixture was autoclaved and incubated (along with suitable controls) so as to simulate experimental conditions.

Growth Studies

Growth curve experiments were run in 250 ml. brown glass bottles fitted with rubber stoppers through which were inserted calibrated Klett tubes. To replicate series of bottles were added:

1. Basal medium, 30 ml.
2. Basal medium, 27 ml.; solution of active fraction (20 mgm./ml.), 3 ml.
3. Basal medium, 29.4 ml.; 1 : 1000 Roccal solution, 0.6 ml. (20 p.p.m.).
4. Basal medium, 26.4 ml.; active fraction, 3 ml.; Roccal solution, 0. 6 ml.
5. Basal medium, 26.4 ml.; Roccal solution, 0.6 ml.

After the components had been added, the bottles were autoclaved, cooled, and inoculated. At periodic intervals the bottles were inverted and the turbidities measured in the Klett Colorimeter. After 8.25 hr., 3 ml. aliquots of the active fraction or sterile water (in the controls) were added and the turbidity readings continued.

Results and Discussion

It can be seen from Fig. 1 that yeast extract interferes with the Roccal inhibition of *S. cerevisiae*. The organism grown in the absence of yeast extract was inhibited by 8 p.p.m. of Roccal, while in the presence of yeast extract, it was able to tolerate 26 p.p.m. of Roccal. In an attempt to determine the distribution of this protective substance, a large number of materials were tested for protective activity (Table I). Among these, only Bacto liver and Bacto beef extract exhibited protective activity. In both cases, the activity was considerably less than that shown by yeast extract.

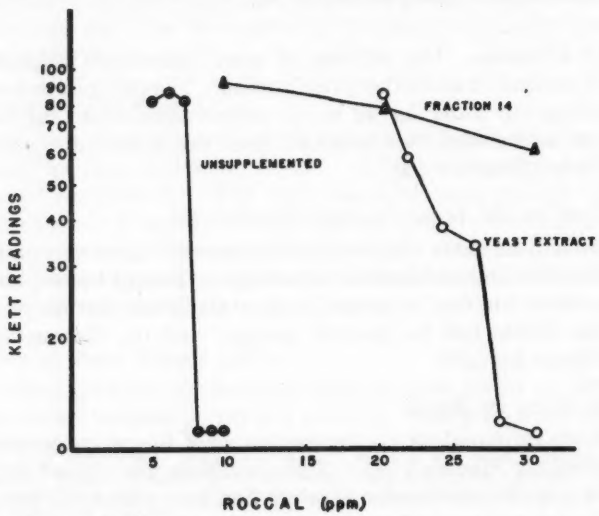

FIG. 1. The inhibition of *S. cerevisiae* by Roccal in the presence and absence of yeast extract (5%) and the active fraction (Fraction 14) 0.01%.

Since maximal activity was demonstrated by yeast extract, it seemed desirable to concentrate the active component found in this material by means of a series of fractionation procedures. After unsuccessful attempts to obtain an active fraction by ether extraction, dialysis, and alcohol precipitation, the yeast extract was precipitated with ammonium sulphate. While protective activity could not be demonstrated with the supernatant, the precipitate showed 60% of the original activity. Subsequent modification of the procedure employed in harvesting the precipitate resulted in the retention of 92% of the original activity. Further, the removal of ammonium sulphate by dialysis and drying at 80° C. for 30 hr. resulted in no loss of activity. The material thus obtained represented a 25-fold increase in protective activity when compared with yeast extract (on a dry weight basis).

In an attempt to characterize the active material, a series of chemical tests were carried out. A micro-Kjeldahl determination indicated a nitrogen content of 6%. Negative ninhydrin and biuret tests precluded the presence of either proteins or peptides. A positive Millon test indicated the presence of phenolic groups, while the absence of desoxyribonucleic acid was indicated by a negative diphenylamine test. A negative Benedict test showed that the active material did not contain reducing carbohydrate.

Up to this time no use has been made of the term *reversal of inhibition*. This has been a purposeful omission, a deliberate evasion of the concept of reversal. The results described to this point reveal nothing as to the mechanism involved in the diminution of Roccal inhibition. It was necessary to consider the possibility that the yeast extract combined directly with Roccal, rendering it inactive. If this were the case, the initial inhibitory concentration might be reduced to a noninhibitory one whereas, in the unsupplemented control, inhibition would still occur. To ascertain if this were so, it was necessary to separate the Roccal from the yeast extract and determine its residual potency. Since a physical separation of the two without molecular alteration would be difficult, a chemical assay of active Roccal in the presence of yeast extract was conducted. The colorimetric assay was performed on a solution of basal media containing 30 p.p.m. of Roccal and 5% yeast extract. This assay is based on the ability of *uncombined* Roccal to form colored salts with tetrabromophenolsulphonphthalein (bromphenol blue). At the end of 48 hr., the active Roccal concentration was unchanged, indicating that the yeast extract did not irreversibly bind or inactivate the Roccal to any significant extent. This conclusion is based on the assumption that the assay is sensitive only to uncombined or active Roccal as suggested by Auerbach (1). It follows, therefore, that the active component of yeast extract does not combine with Roccal so as to render it inactive, but interferes in some manner with the mechanism of inhibition. This appears to be indicative of a true reversal.

Evidence to support this view was obtained by means of growth studies under a variety of conditions. By comparing the growth curves of *S. cerevisiae* in the presence and absence of Roccal it was possible to demonstrate that the

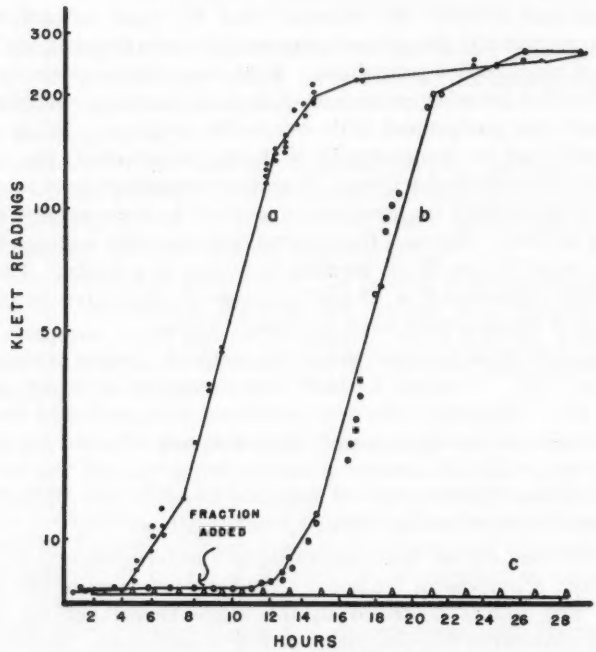

FIG. 2. Roccal inhibition of *S. cerevisiae* reversed by Fraction 14 derived from yeast extract. (*a*) Basal medium plus Fraction 14; (*b*) basal medium plus Roccal with addition of Fraction 14 after eight hours; (*c*) basal media plus Roccal with the addition of sterile water after eight hours.

concentration of Roccal employed in this study completely inhibited growth of the organism (Fig. 2). Further, the addition of the active fraction in a concentration of 2 mgm./ml. eight hours after an inhibitory concentration of Roccal was added reversed the inhibition. There can be no doubt that the rise in curve *a*, representing yeast growth in the absence of Roccal, and the lack of growth indicated by curves *b* and *c*, which represent basal media to which Roccal had been added, are evidence that inhibition had already taken place when the reversing factor was added (Fig. 2). An aliquot removed from the Roccal-containing series and transferred to Roccal-free basal medium exhibited no growth despite the fact that the Roccal in the aliquot was diluted far below (to 0.4 p.p.m.) the effective inhibitory concentration. From this, one might speculate that the quaternary ammonium compound and the microorganism are involved in a tight combination and cannot be separated except by a specific reversing agent.

It might be of interest to reconsider, in the light of the evidence presented in this study, the hypothesis concerning the modes of quaternary ammonium inhibition. If inhibition is considered to be due to an interference with some vital metabolic function, one might expect that inhibition could be reversed

by: (*a*) the addition of metabolites which could replace those lost during disruption of cell permeability, (*b*) a substance capable of removing the inhibitor, allowing metabolic processes to resume, (*c*) a substance supplying the product whose synthesis is blocked, (*d*) a substance capable of directing the organism to an alternative metabolic pathway.

Hotchkiss (7) and Gale and Taylor (4) presented evidence to indicate that seepage of various metabolic products from cells treated with inhibiting concentrations of quaternary ammonium compounds occurred. The similarity between the uninhibited and reversed growth curves (slopes of log phase, and duration of lag periods) precludes the probability of such a mechanism being operative in the present investigation.

Data have been presented to show that an irreversible affinity does not exist between Roccal and yeast extract. Therefore, one would hardly expect that yeast extract could remove the inhibitor from an enzyme protein (for which quaternary ammonium compounds are known to have a strong affinity). Thus, it would appear that, if the inhibition were concerned with metabolic function, the reversing factor must supply the missing material or enable the cell to circumvent the block via an alternative metabolic pathway. It is conceivable that the active fraction and certainly the complete yeast extract are of sufficient complexity to supply those substances whose synthesis may have been blocked.

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